ANNUAL 2013
OF THE CROATIAN ACADEMY
OF ENGINEERING

CROATIAN ACADEMY OF ENGINEERING

Annu. Croat. Acad. Eng.

ISSN 1332-3482

ANNUAL 2013 OF THE CROATIAN ACADEMY OF ENGINEERING

Editor-in-Chief
Zlatko Kniewald

Zagreb, 2014

Published by

Croatian Academy of Engineering, (HATZ)
28 Kačić St., 10000 Zagreb,
Croatia
Prof. Vladimir Andročec, Ph.D.
President of the Croatian Academy of Engineering (HATZ)

Editor-in-Chief

Prof. emer. Zlatko Kniewald, Ph.D., member emeritus of HATZ

Editorial Board

Prof. Marijan Bošnjak, Ph.D., member emeritus of HATZ
Prof. Daslav Hranueli, Ph.D., member of HATZ
Prof. Jasna Kniewald, Ph.D., member emeritus of HATZ
Prof. emer. Zlatko Kniewald, Ph.D., member emeritus of HATZ
Prof. Milena Mandić, Ph.D., member of HATZ
Prof. Božidar Šantek, Ph.D., member of HATZ
Prof. Drago Šubarić, Ph.D., member of HATZ
Prof. Jagoda Šušković, Ph.D., member of HATZ

Technical Editor

Prof. Jasna Kniewald, Ph.D., member emeritus of HATZ

Cover Design

Assist. prof. Ivana Žiljak Ph. D.

ISSN 1332-3482
Annual 2013 of the Croatian Academy of Engineering
Annu. Croat. Acad. Eng.

Prepress
Vladimir Pavlić, GRAPA, Zagreb

Printed by

DENONA, d.o.o.

Circullation

300

Contents

Part II
Presentations

Our forthcoming meeting:
The 3rd International Scientific Symposium "Vera Johanides" – Biotechnology, Forestry and Wood Technology in Croatia, Zagreb, 2015

Part III
Novel Technologies

Letter of HATZ President

Dear readers,

The purpose of this latest edition of the traditional Annual of our Croatian Academy of Engineering (HATZ) is to provide an overview of activities in 2013.

HATZ is a scientific institution founded with the basic task to gather distinguished and most eminent scientists in the field of engineering and biotechnical sciences at the scientific and highly professional level so that they are promoted within the scientific society, high education and business of the Republic of Croatia. This especially applies to the implementation of knowledge, technological achievements and innovations from the academic society into real economy.

With this purpose in mind HATZ momentarily gathers more than 280 members from home and abroad along with about 50 supporting corporative institutions and prominent companies. They are divided into 14 vocational departments in which activities related to presentations, scientific and professional conferences, publishing scientific and professional papers and other publications are carried out individually or in groups. The members of the Academy also work on a series of scientific and professional projects; they promote new technologies and environmental protection and transfer knowledge with the aim of raising the engineering, intellectual and economical level of the Republic of Croatia.

In terms of content this Annual 2013 is focused on the efforts of the members of the Department of Bioprocessing Engineering and other associates who organized the 2nd International Symposium "Vera Johanides" in 2013, which was devoted to Prof. Emerita Vera Johanides, a very distinguished Croatian scientist in the field of biotechnical sciences.

Observing the quality of the presented Symposium articles and the significance of biotechnical sciences, which are especially trend-setting, the HATZ management made a decision to publish its Annual in terms of content as a reviewed overview of the published Symposium papers with two additional papers from this field of our members submitted in the meantime.

I believe that this Annual 2013 will serve the reader as a source of new information on the state and perspectives of biotechnical sciences, and the published results provide the basis for further work.

I thank the Editorial Board headed by the editor-in-chief Prof. Emeritus Zlatko Kniewald for efforts made so that this Annual will be published at an elevated technical level which confirms HATZ even more as an institution promoting scientific and professional achievements of our intellectuals.

Zagreb, March 2014

President of the Croatian Academy of Engineering
Prof. *Vladimir Andročec*, Ph.D.

Letter of the Rector of University of Zagreb

The University of Zagreb has a long and rich history in the field of bio-sciences, with the first fundaments being established as university disciplines in the second half of nineteenth century. Hundred and fifty years later the University is present at the global frontlines of fundamental research, innovations, technology transfers, and study programmes at all stages in the wide spectrum of scientific activities from basic biochemistry, biophysics and molecular biology to biomedicine, life sciences, biotechnologies, bioenergetics, etc. All these fields contribute considerably to its international visibility and the reputation.

With such continuous strengthening of its potentials the University is developing even more ambitious projections for future decades. They are symbolized by two breakthrough projects devoted to its space enlargement.

The first one is the completion of North campus at Šalata and Horvatovac, which will be enriched by the replacement of other natural and biomedical disciplines, besides those already situated there. In particular this enrichment will include the Centre for New Materials and Nano-technology, organized in collaboration with the Institute Ruđer Bošković and the Institute of Physics, as well as the Biomedicine Translational Research Centre which will link the basic and applicative research with the clinical work at the future University Hospital.

The second project is the new Eastern campus in the vast space of Borongaj. It will encompass in particular the majority of life science and biotechnology disciplines, and will be organized as the strong interdisciplinary combination of fundamental and technological research and accompanying studies. It is important to point out that the project of the future Eastern campus itself is in many aspects an interdisciplinary scientific venture which includes globally frontal contemporary issues covering urbanization of modern cities, sustainable and renewable energy and water consumption, new materials and methods in civil engineering including green technologies, new approaches to the culture of living, and many other topics.

The first step in this epochal programme is the building of the new Bioscience Technology Commercialization and Incubation Centre, elaborated in detail in the first article of the present Annual. This Annual of Croatian Academy of Engineering includes also other presentations from the 2nd International symposium “Vera Johanides”, organized by the Academy, and hosted by the University of Zagreb, in May 2013.

On behalf of the University of Zagreb I would like to recall our long and fruitful collaboration with the Croatian Academy of Engineering in the recent years, and to express the gratitude to numerous distinguished members of the Academy, many of them being also the professors of our University, who contributed to this collaboration and to the very remarkable activities of the Academy in the last two decades. Our joint efforts in the successful organization of the 2nd Symposium illustrate in the best possible way the closeness and the mutual interests and preoccupations of our two institutions. They also represent the firm basis for our future joint plans and projects, aiming to contribute further to the development of Croatian science and technology. There is no doubt that the University of Zagreb will continue to contribute in many ways in these endeavours.

Zagreb, 2 March 2014. Prof. *Aleksa Bjeliš*, Ph. D.

Introduction of the Annual 2013

The Croatian Academy of Engineering was established in 1993 with the aim and vision:

- to be the leading creative and innovative multidisciplinary community of scientists in the field of engineering,
- to contribute with excellence and effectiveness to the development of engineering and biotechnological sciences and to the transfer of technological knowledge important for the welfare and progress of the Croatian economy and the benefit of people,
- to advocate safe and appropriate use of technology, environmental protection and the protection of people against an inappropriate use of technology, to promote professionalism and responsible behavior with regard to high ethical norms.

In 2013, the Croatian Academy of Engineering celebrated its 20 years of acting on the realization of the established aims. This year the Republic of Croatia became full member of the European Union and affirmed its historical affiliation to the European community of free and democratic nations.

Today a widely accepted notion is that the 20th Century has been a century of physics and electronics, while the 21st Century is a century of biology and its application in various areas of biotechnology. According to all indicators biotechnology is currently economically more important than physics/microelectronics through available funds, number of employees, number of major discoveries as well as the number of established small and medium enterprises, both in Europe and worldwide. One especially important project for Croatia is the ENPI Horizon 2020 Capacity Building/ Mediterranean Environment Programme (H2020 CB/MEP).

Biotechnology in this region has been conceived as early as 1956 by the establishment of the higher educational Study

of Biotechnology at the former Technological Faculty of the University of Zagreb, with cooperation and for meeting the exigencies of the pharmaceutical and fermentative industries. Twenty years had passed since first ideas and demands for experts in the field of biotechnology to its international recognition as a high technology, the application of which shall in the future change many areas of human activity. At the 1st European Congress of Biotechnology, which had taken place on September 25, 1978 in Interlaken, Switzerland, the Foundation Document of the European Federation of Biotechnology has been signed. Among the first signatories was Professor Vera Johanides, Ph. D., President of the Association of the Yugoslav Societies of Microbiology and President of the Croatian Society of Microbiology. Since the establishment of the Croatian Academy of Engineering in 1993, Professor Emeritus Vera Johanides has been Honorary Member of the Academy until her demise in 2000 at the age of 83. She was a distinguished scientist who, aside from her devoted work as a pedagogist who raised young experts at graduate and postgraduate studies, also realized an excellent cooperation with the industry in the fields of antibiotics production, fermentation processes and environmental protection. By her patents she greatly contributed also to the intellectual property protection. Therefore the Academy named by Professor Vera Johanides its annual award to the young scientists who are not Academy members. The annual awards (five at the most) are granted regularly since 2003, and funds are provided by the Croatian industry.

On September 28, 2011, the Academy had organized the 1st International Symposium by which the achievements of Professor Emeritus Vera Johanides have been revived. On that occasion the Academy had set up the memorial bust of this distinguished scientist.

In the previous period the Croatian Academy of Engineering, in cooperation with the Croatian Society of Biotechnology, has organized five international conferences in English and published their respective proceedings: Biotechnology and Biomedicine (1999), Biotechnology and Environment (2001), Biotechnology and Food (2003), Biotechnology and Immuno-Modulatory Drugs (2005) and Biotechnology, Energy, Chemicals and Renewable Raw Materials (2007).

Starting from all aforementioned and recognizing exceptional significance of biotechnology, we, the biotechnologists, have reached a conclusion that we will organize our regular meetings biannually, addressing the issues of developmental directions and strategical investments in the fields of application of biotechnology in Croatia as follows:

- primary and secondary food and beverages production – green biotechnology,
- health care and pharmaceutical industry – red biotechnology,
- biotechnology of water resources, marine biotechnology and environmental protection – blue biotechnology

- production of industrial raw materials, chemicals, materials and fuels – white biotechnology.

The 2nd International Symposium "Vera Johanides" organized at the Great Hall of the University of Zagreb, on May 10-11, 2013 was directed to:
- strategical directions of the development of Biotechnology in Croatia by 2020,
- economy,
- bioinnovation cores and technological centers,
- bioscience and higher education.

Members of the Organizational and Scientific Committee of the International Symposium were: Prof. Emer. Zlatko Kniewald (Chairman), Prof. Višnja Bačun-Družina, Ph. D., Prof. Marijan Bošnjak, Ph. D., Prof. Jasna Franekić, Ph. D., Prof. Višnja Gaurina Srček, Ph. D., Prof. Ivica Grbac, Ph. D., Prof. Predrag Horvat, Ph. D., Prof. Daslav Hranueli, Ph. D., Prof. Mirjana Hruškar, Ph. D., Prof. Damir Ježek, Ph. D., Prof. Jasna Kniewald, Ph. D., Prof. Milena Mandić, Ph. D., Prof. Vladimir Mrša, Ph. D., Prof. Peter Raspor, Ph. D. (Slovenia), Prof. Anita Slavica, Ph. D., Prof. Božidar Šantek, Ph. D., Prof. Drago Šubarić, Ph. D., Prof. Jagoda Šušković, Ph. D., Prof. Vito Turk, Ph. D. (Slovenia), Prof. Vesna Zechner-Krpan, Ph. D.

This Annual 2013 presents several reviewed papers (part one) and other symposiums powerpoint presentations (part two) that has been already visible through HATZ web sites and the announcement of our forthcoming meeting: The Third International Scientific Symposium "Vera Johanides 3" – Biotechnology, Forestry and Wood Technology in Croatia, Zagreb, 2015.

All powerpoint presentations presented on the 2nd International Symposium "Biotechnology in Croatia by 2020" are available also on the HATZ web sites http://www.hatz.hr/VJ2/prezentacije.php.

For all submitted papers and powerpoint presentations authors are responsible for English expression.

The third part of this Annual is dedicated to the world's major works of our authors for the future.

I would like to thanks to all above mentioned scientists as well as to all speakers for presentations and participants on the meeting for they fruitful discussions, and as well as suporting institutions for financial participation.

Chairman:
Prof. emer. *Zlatko Kniewald*
Member emeritus
of the Croatian Academy of Engineering

The Reception for the Symposium Delegation at the University of Zagreb

Symposium Delegation met at the University of Zagreb with Croatian President Ivo Josipović (center) and the president of the Republic of Slovenia, Borut Pahor (left), Peter Raspor from Slovenia (right)

Rector of the University of Zagreb Aleksa Bjeliš talking to a delegation of the Symposium and Slovenian President Borut Pahor

Members of the delegation in a pleasant conversation with Slovenian President Borut Pahor

Part I

Paper works

BIOCentre: The Bioscience Technology Commercialisation and Incubation Centre

Aleksander Bakowski[1*], Daslav Hranueli[1], Asier Fernández-Matamoros Olaizola[1], Dijana Kobas Dešković[1], Ivana Žorž[2], Gordan Leskovar[2], Srđan Novak[3] and Ivo Friganović[2]

Strategy Paper

[1] ACE International consultants, Calle Sagunto 17 28010 Madrid, SPAIN

[2] BICRO Business Innovation Croatian Agency, Planinska 1, 10000 Zagreb, CROATIA

[3] Centre for research, development and TT, University of Zagreb, Zvonimirova 8, 10000 Zagreb, CROATIA

1. What is BIOCentre?

BIOCentre (Bioscience Technology Commercialisation and Incubation Centre) is technology transfer and commercialization facility in the field of biotechnology and life sciences that will serve academic institutions in order to facilitate biotechnology/life science industry, innovation, competitiveness and to contribute to sustainable regional development. It will look as shown on Fig. 1. BIOCentre will be managed by BICRO BIOCentre Ltd. company which was founded by the Business Innovation Center of Croatia – BICRO, the University of Zagreb and the City of Zagreb. The Business Innovation Center of Croatia – BICRO Ltd. holds the 60% of company shares, the University of Zagreb – 27% and the City of Zagreb – 13%. By linking science and business in one of the most propulsive technologies, the BIOCentre will contribute to industry competitiveness of high value added sectors of the Croatian economy and emergence of new knowledge-based SMEs. The biosciences technology transfer and business incubation facility is a project with a specific aim to develop a high-technology incubation and business development support institution that will create a network of all necessary knowledge, skills and corresponding material resources. Its specific objectives are:

- development of common technical infrastructure that will support biotech start-up businesses, and
- development of service programmes for biotech start-ups covering incubation, contract research and associated service portfolio.

In total, the BIOCentre will have about 5,330 m^2 of gross floor area and about 4,457 m^2 of usable floor area. The spaces are planned with enough flexibility that after the construction of the Centre will be completed the allocation of the spaces

[*] Corresponding author: aleksander.bakowski@bicro.hr

Fig. 1 – Schematic visualisation of the BIOCentre.

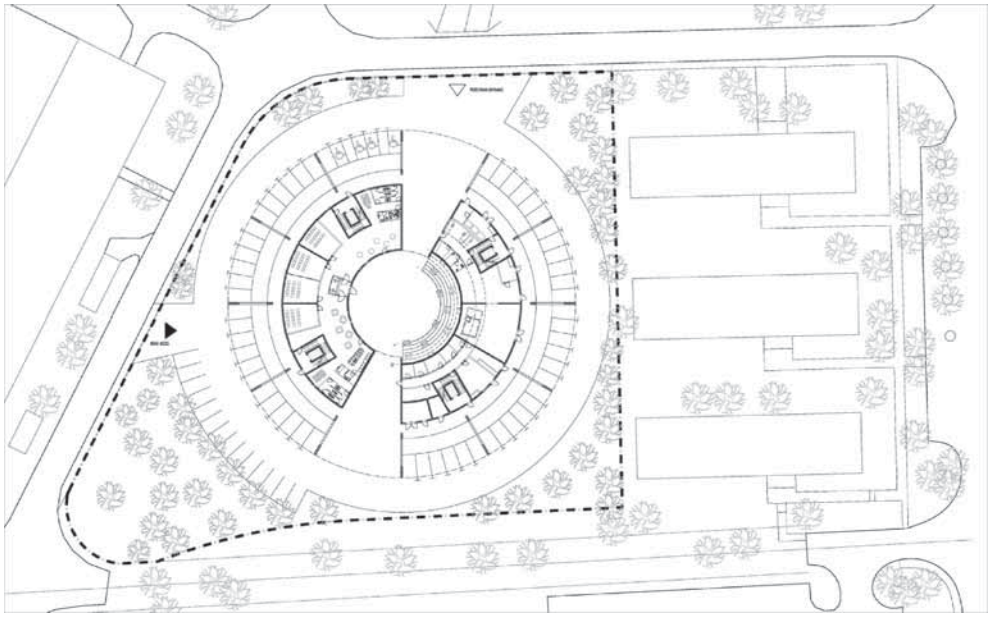


Fig. 2 – Graphical plan of the entire BIOCentre.

may vary and be adapted to the final needs of the companies (Fig. 2). The floor space of the BIOCentre is divided into four categories, according to use:

- laboratory space for tenant companies (equipped by tenant companies according to their needs),
- central laboratory unit (CLU) that consists of seven individual labs (with storage rooms, sterilization and wash room) connected by a safety entrance corridor,
- offices for tenant companies, and
- common spaces (reception, administration area, meeting rooms, conference rooms, social rooms).

Central Laboratory Unit (CLU) will be located on the 2nd floor of BIOCentre and will occupy about 910 m^2 of floor space (including offices) (Fig. 3). Fully equipped CLU will provide contract research services. The CLU will comprise different laboratories and accompanying technical rooms and services:

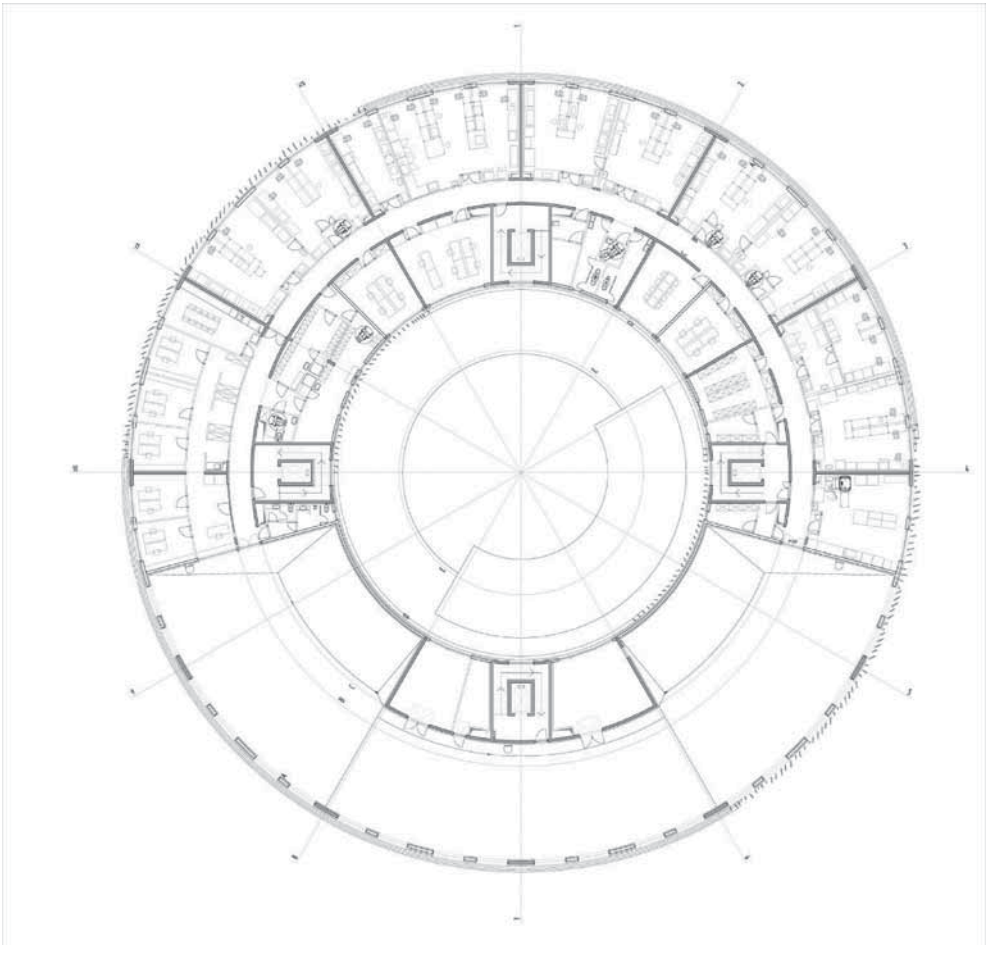


Fig. 3 – Graphical plan of the 2nd floor of the the BIOCentre building.

a. cell banking unit (CBU)
b. cell culture laboratory (CCL)
c. microbial culture laboratory (MCL)
d. product recovery unit (PRU)
e. purification laboratory (PUL)
f. bio-analytical laboratory (BAL)
g. buffer and media preparation unit (BMU)

The CLU is conceptualized to offer complete process development facilities necessary for bio-product development in line with regulatory requirements necessary to develop, commercialize and register new products which are based on bacterial or animal cells as producing organisms. The process can be developed and up scaled from the laboratory to the semi pilot plant scale using laboratory modules: fermentation (e.g. using bioreactors from 7 to 40 L), purification (e.g. using filtration and low pressure liquid chromatography systems), product recovery (e.g. using tangential micro/ultrafiltration system and high pressure homogenizer) and characterisation via bio-analytical procedures (e.g. using gel electrophoresis, UV/VIS spectrometry, LC-MS, HPLC, glycan profiling via HPAE-PAD and MALDI TOF).

Qualified laboratory spaces will be located on the 1st floor occupying about 1067 m^2. Laboratories will be organized in 20 modules per 50 m^2 each (suitable to divide in 25 m^2 labs if necessary) (Fig. 4). Laboratories will only provide basic utilities, whereas the tenants will need to install their own equipment and arrange laboratory space according to their needs. Laboratories will be accessible by companies at any given time. Office spaces for start-up companies will also be located on the 1st floor occupying about 510 m^2. Offices will be organised in 10 modules per about 50 m^2 each (suitable to divide in 25m^2 offices if necessary) with basic office equipment, telecommunications and hosted IT service. Offices will be able to accommodate up to 15 start-ups with up to 15 employees. Companies will have access to 3 conference rooms and 2 smaller rooms for business meetings, all equipped with AV links, projector and white boards. Common spaces: reception, conference rooms, social rooms and technical service area will be located on the ground floor (Fig. 5).

2. Where is it located?

The location chosen for BIOCentre is in the eastern part of the city, called Borongaj, the site designated for the University campus – east. The BIOCentre will benefit from its integration and cooperation with its immediate environment. The University of Zagreb started building a new University campus at the selected location, and BIOCentre is one of its integrated parts. On site are also the Faculty of Food

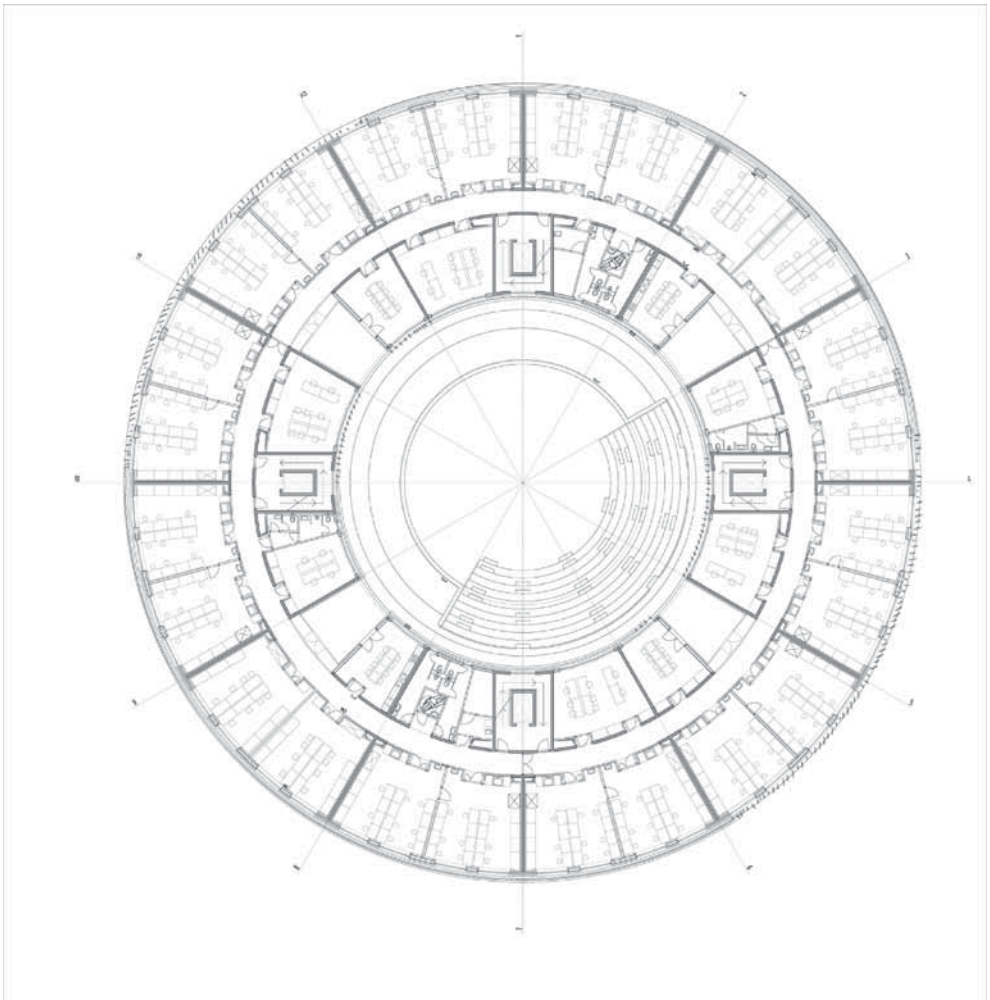


Fig. 4 – Graphical plan of the 1st floor of the BIOCentre building.

Technology and Biotechnology and the Faculty of Chemical Engineering; the Faculty of Agronomy is nearby. The University of Zagreb is the oldest and largest university in South-Eastern Europe. The University has been continually growing and currently comprises 29 faculties with a student body of more than 45 000. By being located within the University campus the BIOCentre will be surrounded by a young vibrant academic atmosphere and will keep close cooperation with the science base. Incoming tenant companies of BIOCentre will also benefit from the academic environment, and vice-versa, the academics may feel motivated by the business-oriented culture created by BIOCentre and its tenants. As the land is well disaggregated, it offers enough possibilities for expansion and is owned by the Ministry of Science, Education and Sports – the land is offered as an additional in-

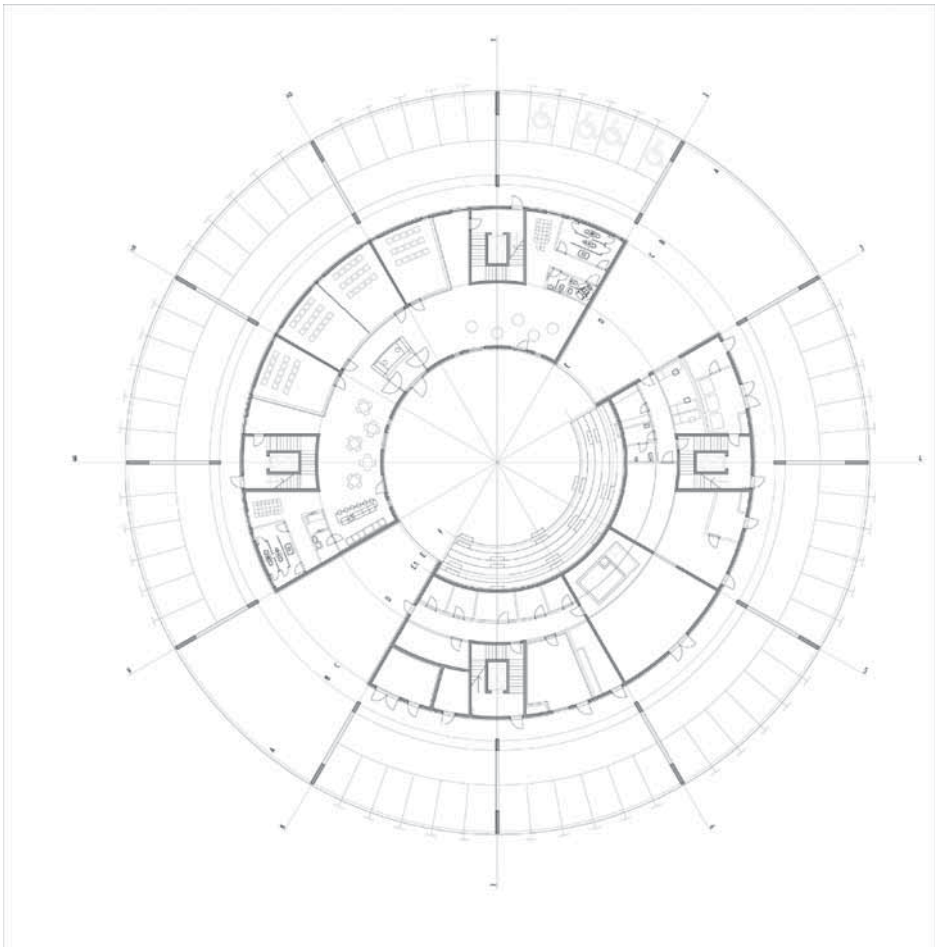


Fig. 5 – Graphical plan of the ground floor of the BIOCentre building.

kind contribution to the Centre – also the sustainability and possible expansion of the BIOCentre is guaranteed at this location.

BIOCentre building is under construction now. Supervision of works by independent consultants and installation of technical equipment will be undertaken during the whole phase of construction (Fig. 6). Laboratory "state of the art" equipment for the CLU has been carefully selected and tender procedure under public procurement regulations is already in process and should be completed at the beginning of next year. Selection of equipment suppliers will be followed by equipment shipment and installation which should be completed by mid of 2014. Final result will be fully equipped building and surrounding infrastructure which will allow easy access to the incubator and laboratories. The majority of the construction and supply costs are financed through the IPA, pre-accession funds.

Fig. 6 – The construction site. View from the crane (A) and from the side (B) (August 2013)

3. What kind of support it offers?

To eliminate the most severe barriers which are faced by biotech start-ups in entering the market: lack of access to scale-up laboratory and professional laboratory staff and lack of skills in business development BIOCentre provides unique approach: incubation functions integrated with a bioprocess development services provided by specialised staff.

The BIOCentre offers:

I. infrastructure to support establishment of biotechnology companies,
II. support in soft services (business advisory and technology transfer know□ how),
III. laboratory infrastructure working according to GLP for biotechnology process development, and
IV. support to access instruments for financing development of new products and processes by companies as well as for financing development of promising young innovative companies, seed and venture financing in particular.

Project development programme will help researchers and entrepreneurs to identify business or project idea, find appropriate source of funding and relevant expertise. Programme will offer also support in project management. BIOCentre incubation programme addressed to tenant companies (start-ups). BIOCentre is an element of biotechnology commercialisation system. It is not a standalone tool but will closely cooperate with other service providers. In that respect the important partners for BIOCentre must be Technology Transfer Offices (TTOs) at the Universities and Public Research Organisations as well as other business support centres (BSC). BSCs are responsible for delivery of an awareness and pre-incubation programme for scientists and other target groups including identification of ideas suitable for commercialisation.

Pre-incubation programme covers:

- "Proof of idea" a process in which technology concept analysis could be performed (technical analysis), its commercial potential could be identified (market needs assessment) and first assessment of whether a person is able to be an entrepreneur should be made (venture assessment). At this stage preliminary commercialization strategy could possibly be defined,
- "Proof of concept" when feasibility of the concept could be analyzed: technical feasibility, market study, business plan for start-up, and
- "Intellectual property right protection" (state of the art search, non-patent publications search, patent search) is a process of relevant documentation analysis and development of strategy for protection and exploitation of IPR.

Incubation programme will secure development of start-up companies when new entrepreneurs have been already identified. The BIOCentre is responsible for provision of high quality business assistance and advisory services to these clients. The BIOCentre staff will tailor their business services to meet the needs of each individual client. During the incubation period, the company is offered services which are essential for improving the skills of managers and employees, market research, promotion and marketing, legal regulations related to the implementation of products/services in different markets, contacts with investors and network of business contacts. The following business assistance and advisory services will be offered as a part of the incubation programme:

- "Proof of business" is process of giving the future entrepreneurs opportunity to test and try out their ideas by creating engineering prototype and develop strategic marketing plan and strategic business plan which includes analysis of the market and competition, as well as the definition of the rights to the patent. A mentoring programme will be gradually developed to secure support by experienced scientific and business mentors, who are able to provide start-up with their experience in the respective field of business, and
- "Business support" is process of supporting start-up company development. It covers early stage investment, assistance in building the management team, coaching, and other advisory services. It will cover also renting of premises in BIOCentre: office space and/or laboratory space.

Addressed to companies business development programme is supporting cooperative R&D projects involving national and international companies and research institutions, with a focus on the up-scaling process of new bio-products. The BIOCentre will offer business development by supporting technology transfer and commercialization through:

- "Process development service" by providing central laboratory services in equipped Central Laboratory Unit (CLU). The CLU is conceptualized to offer complete process development service necessary for bio-product development in line with regulatory requirements necessary to develop, commercialize and register new products. The CLU will be managed by the BIOCentre and staffed with appropriate technical personnel (on average 20 people will be able to work in the CLU). Laboratory covers the whole process development on the lab scale under the GMP conditions in compliance with regulatory requirements and it can be used for the development of the wide range of products for biotech-industry, and
- "Business development services" covering internationalisation (operation on foreign markets), regulatory knowledge, licensing, business transitioning, market validation, as well as support in access to VC funding.

Trainings programme covers business and technical training courses for specific know-how, methods and new technologies. The training programme will provide the following types of training:

- "Business trainings"
  - ✓ Trainings in business development: Training on management, planning, negotiation skills
  - ✓ Trainings in market regulations: Training on regulatory knowledge
- "Technical trainings"
  - ✓ Specialised practical biotech trainings on laboratory techniques and processes
  - ✓ Specialised customised biotech trainings tailored to company needs

"Business trainings" will offer training and support in topics like innovation management, business strategy, market analysis and business financing, legal issues (IPR), development of management, planning and negotiation skills and understanding of the industry. "Technical trainings" will enable the companies staff to receive training and support in process development, GLP (good laboratory practice), GTP (good tissue practice), and GMP (good manufacturing practice) procedures, regulatory compliance, etc. and to get practical knowledge on laboratory and analytical techniques. The courses will be organized and presented together with professional university and industry experts. The scheme of BIOCentre service programme is presented in Fig. 7.

To create positive environment for its operations BIOCentre will build up a network of all institutions, companies and individuals in Croatia which are directly or indirectly connected with the topic of biosciences. BIOCentre will connect domestic network with international counterparts. BIONetwork is also designed to estab-

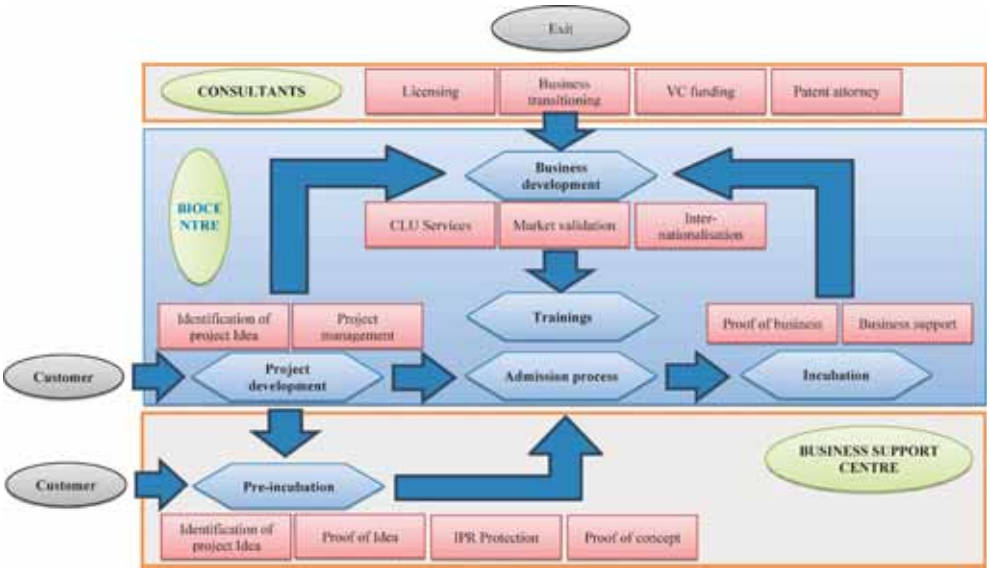


Fig. 7 – The scheme of the BIOCentre service programme.

lish connections between the biosciences community and institutions or potential partners from other sectors – investors, public institutions and business partners from other industries.

4. To whom it offers support?

BIOCentre addresses specialized needs of target groups in the following 5 areas of activity: infrastructure, incubation support, technology transfer, training and networking. The main target groups of the BIOCentre are start-up companies (i.e. spin-offs from universities or other research institutions) operating in the field of biotechnology and life sciences. Moreover, the facility and its services are offered to existing companies and SMEs as well as national and international research institutions and groups for the implementation of innovative projects with significant commercial potential. Preference should be given to projects with strong cooperation between science (university) and business (SME/industry). Business opportunities from multinational companies (industry) are envisioned as well. The BIOcentre addresses its services to:

- Academic start-ups/spin-offs
  Academic start-ups/spin-offs in the field of biotechnology and life sciences are the primary target group to be supported by BIOCentre programmes. The foundation of such start-ups will be facilitated through research funding (aimed at knowledge with a commercial potential), intellectual property protection (with the assistance of technology transfer offices) and the pre-incubation process (undertaken at academic institutions). BIOCentre will be an active participant in these processes, which should lead to a strong deal flow of potential candidates for incubation. Those start-ups that fulfil the admission criteria will be incubated and developed through the BIOCentre programmes. BIOCentre will provide them with infrastructure, training, regulatory knowledge, process development and networking opportunities.

- Researchers looking for opportunities to start their business activities
  Researchers (including doctoral students and interns) that carry out projects with a commercial potential comprise the pool from which future start-ups will be identified, supported and selected for incubation at the BIOCentre. The quantity and quality of the deal flow of the BIOCentre crucially depend on the quality of research, IPR protection and pre-incubation undertaken at academic institutions. Therefore, the universities and research institutions (including their Technology Transfer Offices) should be considered as key stakeholders of the BIOCentre,

which should be targeted through marketing activities and engaged through networking and education activities.

- Companies looking for RTD facilities to develop biotechnology process
  The young innovative companies will be served by some of its services (e.g. education and networking) and process development. The same applies to other SMEs and corporations that find in their interest to use available capacities within the BIOCentre. BIOCentre will primarily attract these companies to use the available RTD services of the BIOCentre through the process development service, as well as to initiate RTD cooperation with research groups.

- Companies looking for high quality specialised training of employees
  Training opportunities will be available to companies through attending the training programme and the BIOCentre will try to attract them as members of the BIONetwork.

- Investors looking for new business opportunities
  Investors which are looking for good projects: VC funds, business angels, corporate investors, cloud investors, are among the main sources of funding for new technology based bio-companies. BIOCentre will attract investors to invest in its tenant companies through BIONetwork.

5. What is its target region?

The main target regions from which clients (in particular start-up companies) are to be recruited are firstly Croatia, secondly South East Europe. None of these regions have developed similar facilities as those which are to be offered through the BIOCentre[1,4]. It is thus expected that the BIOCentre will become a leading bio incubation centre which brings together the best minds in science and business. The BIOCentre will enable the whole process development and product testing under the GLP conditions. Besides the general and specialized laboratory facilities, it will provide biotech start-ups with flexible general infrastructure environment and support services that will enable their fast growth. The central laboratory unit represents the "heart" of the BIOCentre and without this investment it will not be attractive and competitive from an international point of view. Currently no laboratory spaces with such high standard research equipment that can be used by different (external) companies according to their needs are available in Croatia and the neighbouring countries.

6. Conclusions

1. Biotechnology will have a major impact on different industrial branches, primary on pharmaceutical industry, agriculture, food and beverages, chemical industry and environment protection. Those industries have to improve their technology development if they want to maintain their competitiveness on the global market. The demand analysis shows that biosciences build one of the most significant technological strengths in Croatia in terms of knowledge and R&D. Consequently Croatia should consider harvesting this potential for the economic growth and taking efforts to establish itself as a high-tech site in one of the niches of biosciences.

   To make biotechnology industry in Croatia competitive on the global market biotechnology innovation system has to be created that can provide favourable conditions to strengthen the value chain and enable new biotechnology processes and products to be developed by securing a conductive environment for biosciences development (including investments into bio education, competitive science base, industrial RTD programmes and appropriate technology infrastructure). For creation of such system there is a need for a national biotechnology innovation policy with clear policy objectives. Croatia should adjust to the overall trend in biotechnology policy making: to shift from science based to commercialisation based biotechnology policies. Such policy should be based on endogenic strengths[1]. A necessary condition for BIOCentre success is to be located in the heart of innovation policy in Croatia.

   The Croatian Government in medium-term science and technology strategy identified biosciences (biotechnology) among priority sectors, important for the future economic development of Croatia. In practice policy towards biotechnology sector is in Croatia rather generic than biotechnology-specific. Besides BIOCentre („technology infrastructure") there is no other biotechnology specific policy-directed instrument to be implemented in particular regarding „adequate funding resources".

2. Croatia has a significant R&D potential given by the traditionally well-established scientific base, well established educational base (broad range of curricula) in the field of biosciences, numerous initiatives reasonably well-established application industries (in particular, pharmaceutical industry). However, there is a need for development of Strategic RTD Agenda (SRA) for the bio sector in Croatia. This kind of document should be created bottom-up with active participation of all key stakeholders in the sector. In particular the leading role of industry should be accomplished. Well-coordinated and focused strategic RTD programmes should be devised in which BIOCentre role should be defined according to its mission and objectives. BIOCentre should take pivotal position in development of processes and generic technologies and commercialisation

through start-up creation. Successful implementation of strategic programmes should turn high-added value biotechnology based industry into the driving-force of competitive Croatia.

3. At present the biotechnology innovations system in Croatia is not matured enough to develop industrial applications and to apply knowledge for industrial purposes. According to analysis of Croatian biotechnology sector performance in knowledge transmission seems to be very much concentrated on publishing scientific knowledge rather than developing industrial applications and applying for patents[2]. BIOCentre role is to fill the gap between biosciences research and industry in Croatia and to lower existing barriers in commercialisation of research results.

4. Biotechnology has the potential to become a platform for technology advancement that can facilitate transformation and increase productivity of various other 'traditional' industries. These broad applications of biotechnology can thus have strong effects on economic development and growth, as well as on the quality of life. Key stakeholders consider creation of biotechnology platform for communication and exchange of ideas between main stakeholders as a good tool to stimulate sector development. BIOCentre could be used as a networking platform. However, such actions should be supported by a sector oriented policy at a state level[3]. They confirmed the need for RTD projects integrating existing resources and expertise to develop knowledge base in biotechnology. Such integrated projects should be addressed to the thematic areas with strong competence inside Croatia and potential for international cooperation.
   BIOCentre can play important role in implementation of such projects being responsible for process development within central laboratory unit (CLU) and support to commercialisation of results which have market potential and are not going to be commercialised by industrial partners involved.

5. BIOCentre will offer number of services required to support commercialisation process in biotechnology. These services have to be provided in close cooperation with other organisations by defining very clearly the services provided in-house and provided by other organisations.

6. BIOCentre cannot be stand alone "policy instrument" but it should be operating in positive environment for biotechnology processes and products development and commercialisation. It requires implementation of number of policy instruments which will stimulate development of the sector and will create positive impact on economic development. Mix of policy directed instruments should be applied in Croatia to stimulate directly or indirectly the development of the sector. Some of these instruments will have direct impact on BIOCentre activities.
   Several policy-directed instruments have been already implemented in Croatia in previous years. Some of them were discontinued and some are still operational.

Additional instruments have to be implemented to complement the existing ones and to create policy mix for biotechnology commercialisation process. Policy instruments should cover the most critical steps in commercialisation process. Development of such environment will enable BIOCentre to accomplish its mission through growth of a population of biotechnology start-ups and increase of biotechnology companies' competitiveness.

7. To reach the excellence and establish position as a high-tech site in one of the biotechnology niches Croatia needs companies who will transfer the knowledge into market oriented products and services that respond to the international market needs. Such companies, mostly SMEs emerge from the scientific community. In other words, there is a strong need for a common technical infrastructure and service portfolio that will support biotech start-up business in the most critical phase of the development cycle minimizing the risk of investment for entrepreneurs and investors and open the access to the commercial exploitation of the scientific results with biggest market potential. However, in order to maximise the socio-economic benefits of the investment in that infrastructure, the whole range of complementary facilities and services should be provided. Numbers of projects which will use BIOCentre potential for bio-product development have been already identified.

8. Face-to-face consultations covering number of key stakeholders and survey covering about 150 stakeholders have been performed to identify projects with high market potential. Portfolio of potential projects will be created by direct consultations with potential customers. To reach its mission BIOCentre needs number of good ideas for start-ups and proposals for process development generated.

9. BICRO BIOCentre Ltd. will employ two BIOCentre managers shortly: CEO (Chief Executive Officer) and CTO (Chief Technology Officer) . They will gradually implement: Service Programme Plan, Business Plan, Marketing Strategy, HRM Strategy, GLP Procedures and Manuals currently being developed by the team of local and international experts, and negotiate the contracts with potential BIOCentre customers. To make BIOCentre fully operational additional personnel responsible for administration, running the laboratory, and support to start-ups will be gradually employed.

10. It is not reasonable to expect that BIOCentre will become self-sustainable in a short period of time. According to cost-benefits analysis[1] break even (accumulated operating revenues equal operating costs) is foreseen to be reached in 10th year of operation. However, when it comes to socio-economic effects, the BIOCentre project is able to generate significant benefits at reasonable level of risk. High probability of large positive effects in the areas of employment, as well as external and fiscal benefits make establishment of BIOCentre fully justified.

References:

1. BIOCentre Feasibility Study and Cost-Benfit Analysis ver.7.0, Zagreb 2010, unpublished.
2. Iciar Dominguez Lacasa, BioPolis: Inventory and analysis of national public policies that stimulate biotechnology research, its exploitation and commercialisation by industry in Europe in the period 2002-2005: National Report of Croatia, Fraunhofer Institute for Systems and Innovation Research, Karlsruhe, Germany, March 2007
3. Mati, V. Market research: Qualitative on-line survey, TA Project Biosciences Technology Commercialisation and Incubation Centre (BIOCentre), December 2013, unpubluished
4. Olaizola, A.F-M. Documentary search of similar European biocentres, TA Project Biosciences Technology Commercialisation and Incubation Centre (BIOCentre), September 2013, unpublished.

New Approaches in the Development of Antibiotics – The Return of Tetracyclines

Tadeja Lukežič and Hrvoje Petković* *Review*

Acies Bio, d.o.o., Tehnološki park 21, 1000 Ljubljana, Slovenia

Tetracyclines (TCs) are polyketide natural products, which include a number of clinically important antibacterials, such as tetracycline, oxytetracycline (5-hydroxy-TC, OTC) and chlortetracycline (7-chloro-TC, CTC). Second and third generation semi-synthetic derivatives, such as minocycline and doxycycline, have been introduced to the clinic to combat antibiotic resistance. Most recently, tigecycline (9-t-butylglycylamido-minocycline) was introduced, which is the first clinical example of a TC that is functionalized on the C9 of the D ring. TCs can be divided into two groups on the basis of their mode of action, or more correctly on the basis of their ability to inhibit protein synthesis. Tetracycline, oxytetracycline, chlortetracycline, minocycline, doxycycline, and 6-demethyl-6-deoxytetracycline compose a group of effective protein synthesis inhibitors binding to the 30S ribosomal subunit. On the other side, it has been reported that there is a second class of TCs, including chelocardin, anhydrotetracycline, 6-thiatetracycline, anhydrochlortetracycline, and 4-epi-anhydrochlortetracycline, which do not inhibit protein synthesis, thus representing opportunity for the development of novel TCs with different mode of action.
A number of gene clusters encoding biosynthesis of TCs from different actinomycetes have been cloned to date, such as *Streptomyces rimosus* (oxytetracycline,), *Streptomyces aureofaciens* (chlortetracycline, 6-demethylchlortetracycline), *Streptomyces sp.* SF2575 (SF2575), *Dactylosporangium sp.* SC14051 (dactylocycline), and *Amycolatopsis sulphurea* (chelocardin). The biosynthetic pathways and our current understanding on biosynthetic mechanisms involved in the biosynthesis of TCs have significantly improved in the recent years, thus presenting a great potential for generation of medically useful TC analogues by applying biosynthetic engineering approaches.

Key words:

tetracyclines, typical, atypical, chelocardin, polyketide, *Streptomyces*

* Corresponding author: hrvoje.petkovic@aciesbio.com

1. Tetracyclines

The tetracyclines (TCs) are broad-spectrum antibiotics which have been used since the 1940s against a wide range of both Gram-negative and Gram-positive pathogens. Basic TC structure comprises four linearly fused six-membered carbon rings (Fig. 1), to which different functional groups are attached. Clinically used TCs are characterized by a unique C2 amide functionality and one aromatic ring (D), whereas other rings include saturated carbon centers *(1-4)* (Fig. 1). The first generation of TCs includes natural TCs such as tetracycline, oxytetracycline and chlorotetracycline and demethylchlortetracycline, produced by *Streptomyces rimosus* and *Streptomyces aureofaciens* strains, respectively *(2)*. The second generation of TCs includes semi-synthetic derivatives doxycycline and minocycline, exclusively used in human medicine. The need to overcome resistance mechanisms spreading among pathogenic bacteria led to development of third generation of semisynthetic TC analogues – glycylcyclines, of which the most potent analogue is the 9-tert-butylglycylamido derivative of minocycline, also known as tigecycline *(5, 6)*.

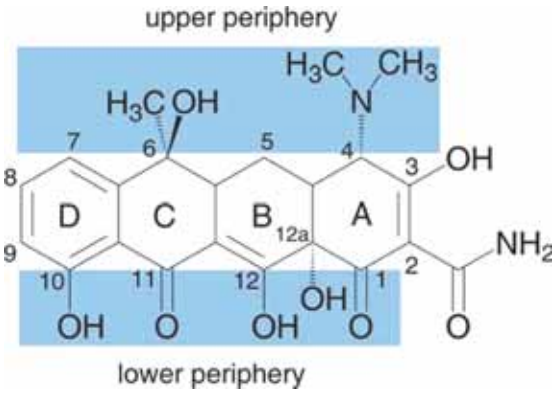


Fig. 1 – TC structure with upper and lower periphery

2. Typical and atypical tetracyclines

TCs can be divided into two groups on the basis of their mode of action, or more correctly on the basis of their ability to inhibit protein synthesis. Tetracycline, oxytetracycline, chlortetracycline, minocycline, doxycycline, and 6-demethyl-6-deoxytetracycline compose a group of effective protein synthesis inhibitors binding to the 30S ribosomal subunit. On the other side, it has been reported that there is a second class of TCs, including chelocardin, anhydrotetracycline, 6-thiatetracycline, anhydrochlortetracycline, and 4-epi-anhydrochlortetracycline, which do not inhibit protein synthesis. TCs from the first group, so-called typical or classic TCs, display a reversible bacteriostatic effect, while the atypical TCs from the second group lead to a strong bactericidal response by

perturbing the organization of the bacterial cytoplasmic membrane *(2, 7, 8)*. Chelocardin is a natural polyketide produced by *Amycolatopsis sulphurea (9)*, whereas other atypical TCs are either chemically synthesized as 6-thiatetracycline *(10)* or shunt products in oxytetracycline and chlortetracycline biosynthesis such as anhydrotetracycline, anhydrochlortetracycline, and 4-epi-anhydrochlortetracycline.

3. Antibacterial activity of typical tetracyclines

Medically important TCs are bacteriostatic agents that inhibit bacterial protein synthesis by preventing the association of aminoacyl-tRNA with the acceptor site of the 30 S subunit of bacterial ribosome *(11, 12)*. Association of TCs with the ribosome is reversible, resulting in their bacteriostatic mode of action. In biological environment typical TCs exist in different conformations: the un-ionized lipophilic form, which can traverse biological membranes, and the zwitterionic form, that predominantly exists in aqueous phases at physiological pH conditions and represents the bioactive form of the antibiotic *(2, 13)*.

TCs traverse the outer membrane of Gram-negative bacteria through the porin channels, as positively charged cation-tetracycline coordination complexes. It is believed that in the periplasm the metal ion-tetracycline (Ca^{2+}) complex dissociates and the uncharged TC is able to diffuse through the lipid bilayer regions of the cytoplasmic membrane. This lipophilic form is also assumed to be the species transferred across the cytoplasmic membrane of gram-positive bacteria. Within the cytoplasm, TC molecules chelate metal ions and most probably one of these, for example magnesium-tetracycline complex, is the form that interferes with ribosome. TCs chelate metal ions with strong affinity and may act as carrier molecules or ionophores, capable of delivering calcium to a host of biological targets, where calcium acts as a secondary messenger, influencing many biological pathways *(2, 4, 13)*. TCs' chelation properties can explain why TCs affect so many different biological targets that are metal-dependent. TCs influence on functioning of ribosomes, transport proteins, metallo-enzymes, secretory processes, receptor activation or inhibition, and cell division cycles. Their ability to bind Ca^{2+} probably causes their undesired inhibitory effect on bone growth and their ability to discolor growing teeth *(13)*. With the use of atypical TCs, such as chelocardin, which exist predominantly in a lipophilic form independently of their enviroment, such unwanted effects could likelly be avoided.

The periphery of the TC molecule can be divided into two distinct regions (Fig. 1), designated the lower and upper peripheral regions, containing different chemical functional groups and substituents. Lower periphery, which extends through oxy-

gen functional groups on carbon atoms C10-C1, and C2 amide functionality are involved in the interaction with the 30S ribosomal subunit. It has been shown that most synthetic modifications along the lower peripheral region of TC greatly decrease biological activity, as both antibiotic and non-antibiotic activity, whereas derivatives modified along the upper peripheral region and on positions C7-C9 demonstrated greater activity against many different biological targets. The C4 dimethylamino group of the molecule is important for its antibiotic activity, however removal of the C4 functional group through synthetic modification of the parent TC increases the activity of these compounds against non-antibiotic targets, such as inhibition of matrix metalloproteinases (MMPs) activity *(2, 3, 13-15)*.

4. Antibacterial activity of atypical tetracyclines

In contrast to the solvent-dependent equilibrium of lipophilic and hydrophilic species of typical TCs, lipophilic conformation of atypical TCs predominates in both polar and nonpolar solvents, which is most probably related to the relative planarity of the B, C, and D rings. On interaction with the cell, the atypical TCs are trapped in the cytoplasmic membrane, therefore disrupting its function. The typical TCs, in contrast, are able to pass through the membrane, without altering any biochemical functions *(2, 7, 8)*, towards the ribosomal cleft, into which TCs fit due to a 'kink' between rings A and B. On the other hand, atypical TCs such as anhydrotetracyclines form more planar molecules, and consequently should not bind to the ribosome.

There were several reports that 6-thiatetracycline and chelocardin act effectively against tetracycline-resistant organisms *(16-20)* and later also Oliva and Chopra *(21)* also demonstrated that Tet resistance determinants provide little or even no protection against atypical TCs, which further supports the division of TCs in above mentioned two groups. Atypical TCs were active against *Escherichia coli* and *Staphylococcus aureus* strains with eflux-mediated resistance (Tet B and Tet K) or ribosomal protection-mediated resistance (Tet M) against typical TCs *(21)*.

Rasmussen and co-workers *(7)* tested the effect of TCs on cell-free translation directed by *Escherichia coli* or *Bacillus subtilis* extracts. They observed that atypical TCs failed to inhibit protein synthesis *in vitro* or were very poor inhibitors in contrast with typical TCs which are very potent inhibitors of protein biosynthesis. However, the atypical TCs rapidly inhibited the *in vivo* incorporation of precursors into DNA and RNA as well as protein, when typical TCs inhibited only incorporation of amino acids into protein *(7)*. Chemical probing methods, used for determination of individual bases in rRNA that are involved in the interaction with the ligand, also confirmed that the ribosome is not the primary target for atypical TCs *(11, 22)*.

Rasmussen et al. *(7)* also suggested that because of the ability to inhibit multiple macromolecular synthetic processes simultaneously, these compounds could interfere with cytoplasmic membrane function. Membrane perturbation prevents substrate accumulation and leads to loss of essential cofactors from the cell. Based on the conformation of atypical TCs, i.e., primarily lipophilic non-ionized form, they suggested that their antimicrobial activity may result from the entrapment of a high proportion of these TC molecules in the cytoplasmic membrane and that the low proportion of molecules that enter the cytoplasm can not contribute to inhibition of bacterial growth. Typical TCs on the other hand, adopting different conformations depending upon their environment, are not trapped in the cytoplasmic membrane and are able to interact with ribosomes *(7)*. Oliva et al. (1992) observed that atypical TCs cause the lysis of *Escherichia coli* accompanied by the release of the cytoplasmic enzyme β-galactosidase into the supernatant. They demonstrated that these compounds caused degenerative changes of cells, including lysis, the formation of vacant ghosts and the release of cellular debris in the culture medium. Although atypical TCs promoted lysis in intact organisms, they did not cause lysis of *E. coli* spheroplasts. They concluded that atypical TCs do not directly destroy the cytoplasmic membrane, but may promote cell lysis by interfering with the membrane's electrochemical gradient, causing membrane deenergization, which could lead to stimulation of autolytic enzyme activity and consequent cellular autolysis *(8)*.

Chelocardin shows potent antibacterial activity agains different Gram-positive and Gram-negative bacteria, including *Clostridium*, *Salmonella*, *Proteus*, *Escherichia coli* and *Staphylococcus aureus* strains, but it is inactive against *Pseudomonas* and fungi *(9, 23)*. Proctor et al. (1978) demonstrated that it is more active against many clinical isolates of aerobic Gram-negative bacilli, but less active against Gram-positive cocci, when compared to TC *(23)*. One of the potential clinical uses of chelocardin could be treating urinary tract infections with for example *Proteus*, *Enterobacter*, *Providencia*, and *Serratia* strains, which are frequently resistant to other agents, but were all susceptible to chelocardin *(23)*. In the seventies chelocardin was subjected to phase II clinical trial for treating urinary tract infections. The results of chelocardin effect on 12 patients were positive and were manifested by the disappearance of clinical symptoms and bacteriuria, accompanied by minor side effects *(24)*.

5. Biosynthesis of tetracyclines

Natural TCs are produced by different strains of the phylum Actinobacteria, such as *Streptomyces rimosus* (oxytetracycline, tetracycline), *Streptomyces aureofaciens* (chlortetracycline, 6-demethylchlortetracycline, tetracycline, 6-demethyltetracycline) *(2)*, *Streptomyces* sp. SF2575 (SF2575) *(25)*, *Dactylosporangium* sp. SC14051

(dactylocycline) *(26)*, *Streptomyces tendae* HKI-179 (cervimycin C) *(27)*, *Amycolatopsis sulphurea* (chelocardin) *(9, 28)*.

5.1 Biosynthesis of clinically used tetracyclines

Early studies on the biosynthesis of chlortetracycline were initialy intensively carried out by McCormick group *(29-38)* at Cyanamid company through feeding experiments of the blocked mutants, producing different shunt products *(29-38)*. The chlorination step was further studied by Dairi et al. *(39)* and recently described in more details by Zhu et al. *(40)*. The oxytetracycline biosynthesis (Fig. 2) on the other hand was intensively studied by research groups in Pfizer and Pliva *(41-46)*, also trying to explain the biosynthesis through the analysis of blocked mutants and molecullar genetics approaches. Many other research groups have contributed to the understanding of oxytetracycline biosynthesis. Petković et al. *(47)* showed the influence of the aromatase/cyclase OtcD1 (OxyK) on the length of polyketide chain, when on the other hand Perić-Concha et al. *(48)* demonstrated that OtcC (OxyS) plays an important role in determining the correct chain length of polyketide product, both experiments implying that these enzymes play a important role in the overall integrity of the quaternary structure of the polyketide synthase complex. Significant part in decoding the TC biosynthesis has been contributed in the recent years by research group of Tang and colaborators *(49-52)* at the University of California, Los Angeles, which described the early tailoring steps of oxytetracycline biosynthesis and the minimal set of enzymes needed for the biosynthesis of anhydrotetracycline *(49)*, explained the C4/C12a oxygenation *(50)* and recently also the C5 oxygenation, a unique step in oxytetracycline biosynthesis *(51)*. Priming in oxytetracycline biosynthesis by unusual starter unit, malonamyl-CoA was also studied by this group *(52)*. Entire chlortetracycline and oxytetracycline (Fig. 3) biosynthetic gene clusters have been cloned and sequenced in 1996 and 2006, respectively *(53, 54)*. A number of regulatory genes, not fosuss of this review have also been identified in gene clusters encoding TC biosynthesis *(55)*.

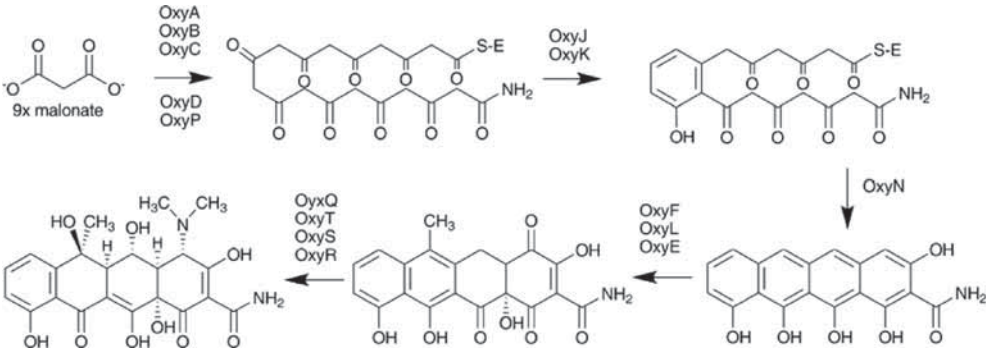


Fig. 2 – Proposed oxytetracycline biosynthetic pathway

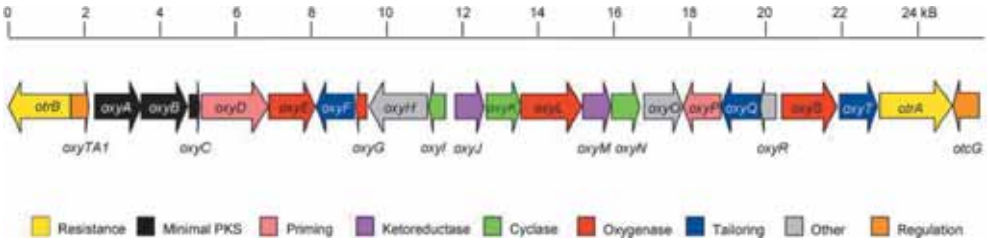


Fig. 3 – Oxytetracycline biosynthetic gene cluster from Streptomyces rimosus

The polyketide skeleton of TCs is synthesized by the type II minimal polyketide synthases (minimal PKS) genes *(5, 56)*, consisting of ketosynthase α, ketosynthase *β* and acyl-carrier protein, CtcW, CtcV, and CtcU in chlortetracycline biosynthesis and OxyA, OxyB, and OxyC in oxytetracycline biosynthesis (Figs. 2 and 3), respectively *(3, 57)*. The minimal PKS enzymes catalyze the iterative Claisen-like condensation of malonamyl starter unit and eight extender malonyl-CoA units, resulting in amidated decaketide backbone, where amidotransferases (CtcT, OxyD) and acyltransferases (OxyP) are responsible for the biosynthesis and the integration of the malonamyl starter unit *(52)*. Correct folding of the polyketide chain is directed by ketoreductases (CtcG, OxyJ), which catalyze region-specific reduction at C8 *(47)* (Figs. 2 and 3). Cyclisation and aromatization is carried out by a number of proteins. Closure of the first (D) ring is catalyzed by two-component cyclases/aromatases (CtcF, OxyK) and closure of the second (C) ring by one-component cyclases (CtcD, OxyN), whereas the closure of the third (B) ring is believed to be spontaneous. Last (A) ring formation was at first suggested to be catalyzed by the third group of cyclases (CtcH, OxyI), but it was later proposed that the closure of the final ring of TCs is spontaneous because of the presence of the terminal amino group *(58)* (Figs. 2 and 3). The post-PKS or tailoring reactions further decorate the TC scaffold. Methyltransferases (CtcK, OxyF) are responsible for C6 methylation *(58)*, which is followed by a double hydroxylation of ring A at C4/C12a by oxygenase pairs like CtcE and CtcX or OxyL in OxyE, where OxyE is believed to be an ancillary mono-oxygenase for OxyL with a nonessential, but important role in improving its efficiency as a C4 hydroxylase *(50)* (Figs. 2 and 3). Hydroxylation at C4 is a prerequisite for amino group incorporation at C4 by PLP-dependent aminotransferases (CtcL, OxyQ), which is followed by dimethylation of the amino group by *N,N*-dimethyltransferases CtcO or OxyT to yield an atypical TC – anhydrotetracycline (ATC) *(49)*. Hydroxylation of ATC at C6 is catalyzed by FAD-dependent monooxygenases (CtcN, OxyS), where OxyS is also responsible for hydroxylation of C5 in the biosynthesis of oxytetracycline *(51)*. Penultimate step in chlortetracycline and last step in oxytetracycline biosynthesis is believed to be reduction of the C5a−C11a double bond by CtcM or OxyR, to yield tetracycline or oxytetracycline (Figs. 2 and 3), respectively *(51)*. Last step in the chlortetracycline biosynthesis, chlorination of C7, is performed by flavin-dependent halogenase CtcP *(40)*.

5.2 Biosynthesis of tetracyclines not used in clinic

Among the TCs, which are not used in clinic, biosynthetic gene clusters of SF2575, dactylocycline, and chelocardin (Fig. 4) have been cloned and sequenced. Their biosynthetic pathways were proposed by comparative studies to the gene cluster encoding oxytetracycline or by succesful expression in a heterologous host *(59-61)*. SF2575 displays potent anticancer activity towards a broad range of cancer cell lines and is characterized by a highly substituted TC aglycon with some unusual modifications, as angelate and salicylate moieties. SF2575 contains two methoxy groups at C-6 and C-12a, a 4-(S)-salicylate that replaces the more common 4-(R)-dimethylamine substituent, is glycosylated at the C-9 of the D-ring with D-olivose, and acylated at the C4'-hydroxyl of D-olivose with angelic acid (Fig. 4) *(59)*. Dactylocycline is active against tetracycline-resistant bacterial strains and is also characterized by unique structural modifications, including C7 halogenation, a C4a hydroxylation, an 8-O methylation, a 6-O glycosylation with a hydroxyamino deoxysugar (Fig. 4) *(60)*.

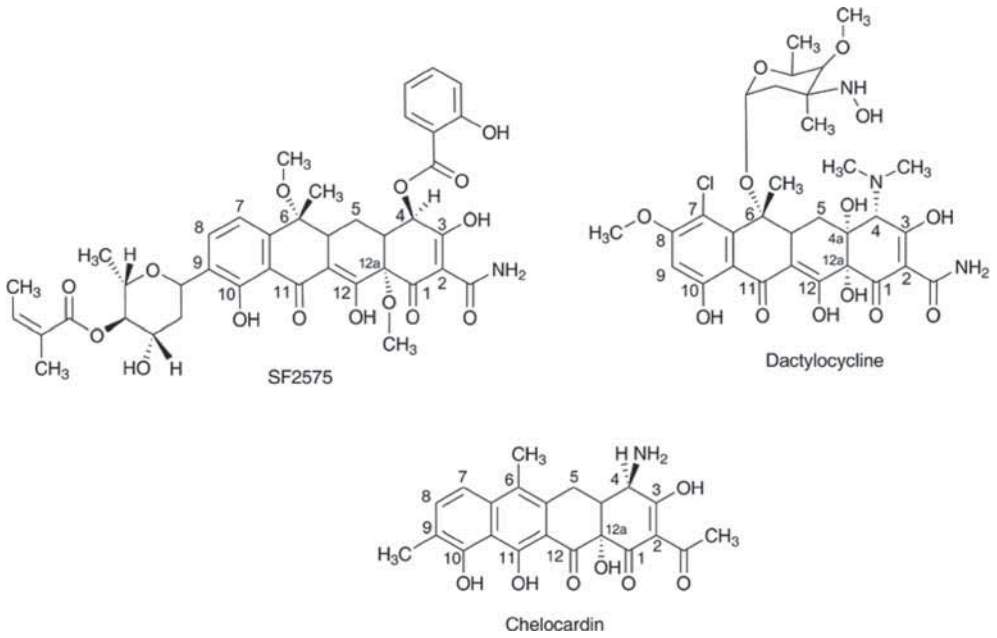


Fig. 4 – Group of tetracyclines, which are not yet in the clinical use

Chelocardin (also cetocycline or M-319) *(9)*, produced by actinomycete *Amycolatopsis sulphurea* (NRRL2822) *(28)* is a potent broad-spectrum atypical tetracycline antibiotic. Its structure was determined in 1970 by Mitscher et al. *(62)*. Chelocardin is 2-decarboxamido-2-acetyl-4-dedimethylamino-4-epi-amino-9-methyl-5a,6-anhydrotetracycline ($C_{22}H_{21}NO_7$) *(62)* and differs from typical TCs in aromatic ring

C, acetyl moiety at C2, non-methylated amino moiety with β-stereochemistry at C4 (all three usually associated with reduced biological potency in typical TCs), and an additional methyl group at C9 (consistent with substantial in vitro potency), where particular combination of the features, normally associated with poor activity in TCs, results in a potent activity of chelocardin *(63)*. Compared to oxytetracycline, chelocardin does not contain hydroxyl groups at C5 and C6 and differs in the pattern of reduction of carboxyl groups at C11 and C12 (Fig 4), thus resulting in the less polar structure of chelocardin.

5.3 Proposed biosynthetic pathway of chelocardin

Mitscher and coworkers *(64)* studied the chelocardin biosynthesis through incorporation experiments using ^{13}C-labeled acetate and determined that in addition to its role as a extender unit precursor, acetate also serves as the starter unit. The chelocardin biosynthetic gene cluster was identified and cloned in recent years (Figs. 5 and 6) *(61, 65, 66)*.

Chelocardin is an aromatic polyketide synthesized by a type II polyketide synthase (PKS). Its polyketide backbone is assembled from 10 malonate-derived building blocks by the putative minimal PKS enzymes, consisting of the ketosynthase α (ChdP), ketosynthase β (ChdK), and the acyl carrier protein (ChdS) (Figs. 5 and 6). Correct folding of the polyketide chain is most likely directed by a putative aromatic PKS ketoreductase ChdT, which catalyzes region-specific reduction at C8 (Figs. 5 and 6). Based on the facts that the *chd* cluster encodes two cyclase/aromatase OxyK homologues, ChdQII and ChdQI and that there are two adjacent aromatic rings (D and C) in the chelocardin molecule, it was proposed that the closure of ring D is catalyzed by the first OxyK homologue – a putative cyclase/aromatase ChdQII *(65, 66)*, whereas the second cyclase/aromatase homologue (ChdQI) may be needed for aromatisation of ring C. It was shown that the formation of ring B in

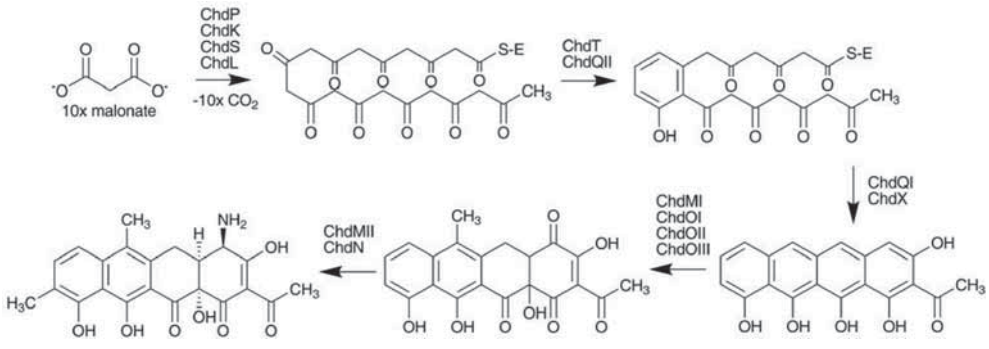


Fig. 5 – Chelocardin biosynthetic gene cluster for Amycolatopsis sulphurea

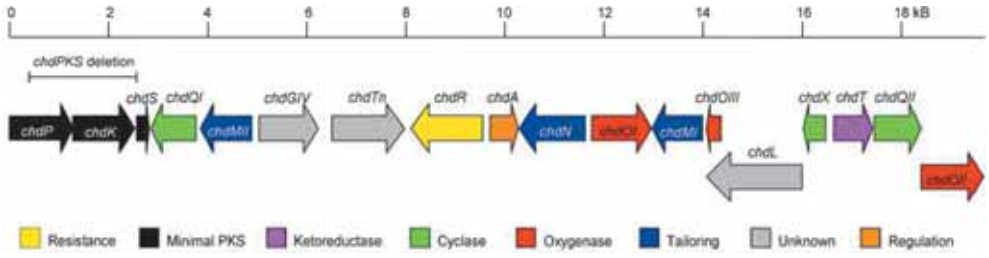


Fig. 6 – Proposed biosynthetic pathway of chelocardin

the biosynthesis of typical TCs is spontaneous *(58)*, which colud also be case in chelocardin biosynthesis. Closure of the final ring (A) is most likely catalyzed by a putative monodomain cyclase ChdX (Figs. 5 and 6), homologous to OxyI from *S. rimosus*. Even though it was shown for OxyI not to have an essential role in oxytetracycline biosynthesis, it was also proposed that monodomain cyclases are needed for closure of ring A of those polyketides that have acetate as a primer unit (such as daunorubicin, tetracenomycin and chelocardin) *(58)*. Post-PKS tailoring reactions of chelocardin include C4/C12a hydroxylation, C6- and C9-methylations, and C4-amination. C4/C12a hydroxylation is most likely carried out by ChdOII, homologous to C4/C12a hydroxylase OxyL from oxytetracycline biosynthesis *(3, 54)* (Figs. 5 and 6). As the mono-oxygenase OxyE in the oxytetracycline biosynthesis, most probably also its homologue ChdOI has a nonessential, but important role in improving the efficiency C4 hydroxylation *(50)*. C6- and C9-methylations are most likely catalyzed by two putative methyltransferases, ChdMI and ChdMII, respectively, the latter representing a unique characteristic of chelocardin, when compared to typical TCs, and most likely contributing to chelocardin's different mode of action. ChdMI and ChdMII putative functions were proposed based on their homology to OxyF (C6-methylase in oxytetracycline biosynthesis) and CmmMII (C9-methyltransferase in chromomycin biosynthesis), respectively *(54, 65-67)*. C4-amination is most likely catalysed by ChdN (Figs. 5 and 6), the only ORF in the *chd* cluster with homology to an aminotransferase. Interestingly, ChdN is unrelated to C4-aminotransferases OxyQ and Ctc16 from the *otc* and *ctc* gene clusters respectively, which may explain the opposite stereochemistry of C4-amino group of chelocardin, when compared to typical TCs.

6. Prospects for new tetracycline antibiotics

The tetracycline scaffold has been a valuable inspiration for medicinal chemists, and tetracycline derivatives still play an important role in treating bacterial infections. Through the identification of gene clusters and study of TC biosynthesis, it is becoming more and more evident what a great biosynthetic potential these diverse

gene clusters can offer, and the opportunities to produce new analogues with improved antimicrobial activity. Identification of the role of individual enzymes in the diverse pathways encoding TC biosynthesis is another step forward toward rational engineering of new TC compounds for generation of medically valuable antibacterials.

References

1. I. Chopra, P.M. Hawkey, M. Hinton, Tetracyclines, molecular and clinical aspects, *J. Antimicrob. Chemother. 29* (1992) 245-77.
2. I. Chopra, M. Roberts, Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance, *Microbiol. Mol. Biol. Rev. 65* (2001) 232-60.
3. L.B. Pickens, Y. Tang, Oxytetracycline biosynthesis, *J. Biol. Chem. 285* (2010) 27509-15.
4. M. Thaker, P. Spanogiannopoulos, G.D. Wright, The tetracycline resistome, *Cell. Mol. Life Sci. 67* (2010) 419-31.
5. C. Hertweck, A. Luzhetskyy, Y. Rebets, A. Bechthold, Type II polyketide synthases: gaining a deeper insight into enzymatic teamwork, *Nat. Prod. Rep. 24* (2007) 162-90.
6. P.E. Sum, V.J. Lee, R.T. Testa, J.J. Hlavka, G.A. Ellestad, J.D. Bloom, Y. Gluzman, F.P. Tally, Glycylcyclines. 1. A new generation of potent antibacterial agents through modification of 9-aminotetracyclines, *J. Med. Chem. 37* (1994) 184-8.
7. B. Rasmussen, H.F. Noller, G. Daubresse, B. Oliva, Z. Misulovin, D.M. Rothstein, G.A. Ellestad, Y. Gluzman, F.P. Tally, I. Chopra, Molecular basis of tetracycline action: identification of analogs whose primary target is not the bacterial ribosome, *Antimicrob. Agents Chemother. 35* (1991) 2306-11.
8. B. Oliva, G. Gordon, P. McNicholas, G. Ellestad, I. Chopra, Evidence that tetracycline analogs whose primary target is not the bacterial ribosome cause lysis of *Escherichia coli*, *Antimicrob. Agents Chemother. 36* (1992) 913-9.
9. T.J. Oliver, A.C. Sinclair, Antibiotic M-319. *US patent 3155582* (1964).
10. W. Rogalski, R. Kirchlechner, J. Seubert, R. Gottschlich, R. Steinigeweg, R. Bergmann, H. Wahlig, J. Gante, Tetracyclic compounds. *US patent 4024272* (1977).
11. D. Moazed, H.F. Noller, Interaction of antibiotics with functional sites in 16S ribosomal RNA, *Nature, 327* (1987) 389-94.
12. M. Pioletti, F. Schlunzen, J. Harms, R. Zarivach, M. Gluhmann, H. Avila, A. Bashan, H. Bartels, T. Auerbach, C. Jacobi, T. Hartsch, A. Yonath, F. Franceschi, Crystal structures of complexes of the small ribosomal subunit with tetracycline, edeine and IF3, *EMBO J. 20* (2001) 1829-39.
13. M.L. Nelson, Chemical and biological dynamics of tetracyclines, *Adv. Dent. Res. 12* (1998) 5-11.
14. B. Zakeri, G.D. Wright, Chemical biology of tetracycline antibiotics, *Biochem. Cell Biol. 86* (2008) 124-36.
15. L.M. Golub, T.F. McNamara, G. D'Angelo, R.A. Greenwald, N.S. Ramamurthy, A non-antibacterial chemically-modified tetracycline inhibits mammalian collagenase activity, *J. Dent. Res. 66* (1987) 1310-4.
16. V. Burdett, J. Inamine, S. Rajagopalan, Heterogeneity of tetracycline resistance determinants in *Streptococcus*, *J. Bacteriol. 149* (1982) 995-1004.

17. Y.A. Chabbert, M.R. Scavizzi, Chelocardin-inducible resistance in *Escherichia coli* bearing R plasmids, *Antimicrob. Agents Chemother. 9* (1976) 36-41.

18. B. Mendez, C. Tachibana, S.B. Levy, Heterogeneity of tetracycline resistance determinants, *Plasmid, 3* (1980) 99-108.

19. A.D. Russell, I. Ahonkhai, Antibacterial activity of a new thiatetracycline antibiotic, thiacycline, in comparison with tetracycline, doxycycline and minocycline, *J. Antimicrob. Chemother. 9* (1982) 445-9.

20. B. Traub, C.F. Beck, Resistance to various tetracyclines mediated by transposon Tn10 in *Escherichia coli* K-12, *Antimicrob. Agents Chemother. 27* (1985) 879-81.

21. B. Oliva, I. Chopra, Tet determinants provide poor protection against some tetracyclines: further evidence for division of tetracyclines into two classes, *Antimicrob. Agents Chemother. 36* (1992) 876-878.

22. S. Stern, D. Moazed, H.F. Noller, Structural analysis of RNA using chemical and enzymatic probing monitored by primer extension, *Methods Enzymol. 164* (1988) 481-9.

23. R. Proctor, W. Craig, C. Kunin, Cetocycline, tetracycline analog: in vitro studies of antimicrobial activity, serum binding, lipid solubility, and uptake by bacteria, *Antimicrob. Agents Chemother. 13* (1978) 598-604.

24. V. Molnar, Z. Matkovic, T. Tambic, C. Kozma, Klinicko-farmakolosko ispitivanje kelokardina u bolesnika s infekcijom mokracnih puta, *Lij. vjes. 99* (1977) 560-562.

25. M. Hatsu, T. Sasaki, H. Watabe, S. Miyadoh, M. Nagasawa, T. Shomura, M. Sezaki, S. Inouye, S. Kondo, A new tetracycline antibiotic with antitumor activity. I. Taxonomy and fermentation of the producing strain, isolation and characterization of SF2575, *J. Antibiot. (Tokyo) 45* (1992) 320-4.

26. J.S. Wells, J. O'Sullivan, C. Aklonis, H.A. Ax, A.A. Tymiak, D.R. Kirsch, W.H. Trejo, P. Principe, Dactylocyclines, novel tetracycline derivatives produced by a *Dactylosporangium* sp. I. Taxonomy, production, isolation and biological activity, *J. Antibiot. (Tokyo) 45* (1992) 1892-8.

27. K. Herold, Z. Xu, F.A. Gollmick, U. Grafe, C. Hertweck, Biosynthesis of cervimycin C, an aromatic polyketide antibiotic bearing an unusual dimethylmalonyl moiety, *Org. Biomol. Chem. 2* (2004) 2411-4.

28. M.P. Lechevalier, H. Prauser, D.P. Labeda, J.S. Ruan, Two new genera of nocardioform *Actinomycetes*: *Amycolata* gen. nov. and *Amycolatopsis* gen. nov., *Int. J. Syst. Bacteriol. 36* (1986) 29-37.

29. J.R. McCormick, E.R. Jensen, Biosynthesis of the tetracyclines. XII. Anhydrodemethylchlortetracycline from a mutant of *Streptomyces aureofacients*, *J. Am. Chem. Soc. 91* (1969) 206.

30. J.R. McCormick, E.R. Jensen, Biosynthesis of tetracyclines. X. Protetrone, *J. Am. Chem. Soc. 90* (1968) 7126-7.

31. J.R. McCormick, E.R. Jensen, Biosynthesis of the Tetracyclines. VIII. Characterization of 4-Hydroxy-6-Methylpretetramid, *J. Am. Chem. Soc. 87* (1965) 1794-5.

32. J.R. McCormick, E.R. Jensen, N. Arnold, H.S. Corey, U.H. Joachim, S. Johnson, P.A. Miller, N.O. Sjolander, Biosynthesis of tetracyclines. XI. The methylanthrone analog of protetrone, *J. Am. Chem. Soc. 90* (1968) 7127-9.

33. J.R. McCormick, E.R. Jensen, S. Johnson, N.O. Sjolander, Biosynthesis of the tetracyclines. IX. 4-Aminodedimethylaminoanhydrodemethylchlortetracycline from a mutant of *Streptomyces aureofaciens*, *J. Am. Chem. Soc. 90* (1968) 2201-2.

34. J.R. McCormick, U.H. Joachim, E.R. Jensen, S. Johnson, N.O. Sjolander, Biosynthesis of the Tetracyclines. VII. 4-Hydroxy-6-Methylpretetramid, an Intermediate Accumulated by a Blocked Mutant of *Streptomyces aureofaciens*, *J. Am. Chem. Soc. 87* (1965) 1793-4.

35. J.R. McCormick, N.O. Sjolander, S. Johnson, A.P. Doerschuk, Biosynthesis of tetracycline's. II. Simple, defined media for growth of *Streptomyces aureofaciens* and elaboration of 7-chlorotetracycline, *J. Bacteriol. 77* (1959) 475-7.

36. J.R.D. McCormick, S. Johnson, N.O. Sjolander, Biosynthesis of the tetracyclines. V. Naphthacenic precursors, *J. Am. Chem. Soc. 85* (1963) 1692–1694.

37. J.R.D. McCormick, P.A. Miller, S. Johnson, N. Arnold, N.O. Sjolander, Biosynthesis of the tetracyclines. IV. Biological rehydration of the 5a,6-anhydrotetracyclines, *J. Am. Chem. Soc. 84* (1962) 3023–3025.

38. J.R.D. McCormick, J. Reichenthal, S. Johnson, N.O. Sjolander, Biosynthesis of the tetracyclines. VI. Total synthesis of a naphthacenic precursor: 1,3,10,11,12-pentahydroxynaphthacene-2-carboxamide, *J. Am. Chem. Soc. 85* (1963) 1694–1695.

39. T. Dairi, T. Nakano, K. Aisaka, R. Katsumata, M. Hasegawa, Cloning and nucleotide sequence of the gene responsible for chlorination of tetracycline, *Biosci. Biotechnol. Biochem. 59* (1995) 1099-106.

40. T. Zhu, X. Cheng, Y. Liu, Z. Deng, D. You, Deciphering and engineering of the final step halogenase for improved chlortetracycline biosynthesis in industrial *Streptomyces aureofaciens*, *Metab. Eng. 19* (2013) 69-78.

41. P.M. Rhodes, N. Winskill, E.J. Friend, M. Warren, Biochemical and genetic characterization of *Streptomyces rimosus* mutants impaired in oxytetracycline biosynthesis, *Microbiology, 124* (1981) 329-338.

42. M.J. Butler, C. Binnie, I.S. Hunter, D.A. Sugden, M. Warren, Genetic manipulation of the oxytetracycline biosynthetic pathway genes, *Dev. Ind. Microbiol. 31* (1990) 41–50.

43. C. Binnie, M. Warren, M.J. Butler, Cloning and heterologous expression in *Streptomyces lividans* of *Streptomyces rimosus* genes involved in oxytetracycline biosynthesis, *J. Bacteriol. 171* (1989) 887-95.

44. H. Petkovic, J. Cullum, D. Hranueli, I.S. Hunter, N. Peric-Concha, J. Pigac, A. Thamchaipenet, D. Vujaklija, P.F. Long, Genetics of *Streptomyces rimosus*, the oxytetracycline producer, *Microbiol. Mol. Biol. Rev. 70* (2006) 704-28.

45. J. Pigac, M. Alacevic, Mapping of oxytetracycline genes in *Streptomyces rimosus*, *Period. Biol. 81* (1979) 575-582.

46. K. Pandza, G. Pfalzer, J. Cullum, D. Hranueli, Physical mapping shows that the unstable oxytetracycline gene cluster of *Streptomyces rimosus* lies close to one end of the linear chromosome, *Microbiology, 143 (Pt 5)* (1997) 1493-501.

47. H. Petkovic, A. Thamchaipenet, L.H. Zhou, D. Hranueli, P. Raspor, P.G. Waterman, I.S. Hunter, Disruption of an aromatase/cyclase from the oxytetracycline gene cluster of *Streptomyces rimosus* results in production of novel polyketides with shorter chain lengths, *J. Biol. Chem. 274* (1999) 32829-34.

48. N. Peric-Concha, B. Borovicka, P.F. Long, D. Hranueli, P.G. Waterman, I.S. Hunter, Ablation of the otcC gene encoding a post-polyketide hydroxylase from the oxytetracyline biosynthetic pathway in *Streptomyces rimosus* results in novel polyketides with altered chain length, *J. Biol. Chem. 280* (2005) 37455-60.

49. W. Zhang, K. Watanabe, X. Cai, M.E. Jung, Y. Tang, J. Zhan, Identifying the minimal enzymes required for anhydrotetracycline biosynthesis, *J. Am. Chem. Soc. 130* (2008) 6068-9.

50. P. Wang, W. Zhang, J. Zhan, Y. Tang, Identification of OxyE as an ancillary oxygenase during tetracycline biosynthesis, *Chembiochem, 10* (2009) 1544-50.

51. P. Wang, G. Bashiri, X. Gao, M.R. Sawaya, Y. Tang, Uncovering the enzymes that catalyze the final steps in oxytetracycline biosynthesis, *J. Am. Chem. Soc. 135* (2013) 7138-7141.

52. P. Wang, X. Gao, Y.H. Chooi, Z. Deng, Y. Tang, Genetic characterization of enzymes involved in the priming steps of oxytetracycline biosynthesis in *Streptomyces rimosus*, *Microbiology, 157* (2011) 2401-9.

53. M.J. Ryan, J.A. Lotvin, N. Strathy, S.E. Fantini, Cloning of the biosynthetic pathway for chlortetracycline and tetracycline formation and cosmids useful therein. *US Patent 5589385* (1996).

54. W. Zhang, B.D. Ames, S.C. Tsai, Y. Tang, Engineered biosynthesis of a novel amidated polyketide, using the malonamyl-specific initiation module from the oxytetracycline polyketide synthase, *Appl. Environ. Microbiol. 72* (2006) 2573-80.

55. U. Lesnik, A. Gormand, V. Magdevska, S. Fujs, P. Raspor, I. Hunter, H. Petkovic, Regulatory elements in tetracycline-encoding gene clusters: the *otcG* gene positively regulates the production of oxytetracycline in *Streptomyces rimosus*, *Food Technol. Biotechnol. 47* (2009) 323-330.

56. D.A. Hopwood, Genetic contributions to understanding polyketide synthases, *Chem. Rev. 97* (1997) 2465-2498.

57. M.J. Ryan, Strain for the production of 6-demethyltetracycline, method for producing the strain and vector for use in the method. *US Patent* (1999).

58. W. Zhang, K. Watanabe, C.C. Wang, Y. Tang, Investigation of early tailoring reactions in the oxytetracycline biosynthetic pathway, *J. Biol. Chem. 282* (2007) 25717-25.

59. L.B. Pickens, W. Kim, P. Wang, H. Zhou, K. Watanabe, S. Gomi, Y. Tang, Biochemical analysis of the biosynthetic pathway of an anticancer tetracycline SF2575, *J. Am. Chem. Soc. 131* (2009) 17677-89.

60. P. Wang, W. Kim, L.B. Pickens, X. Gao, Y. Tang, Heterologous expression and manipulation of three tetracycline biosynthetic pathways, *Angew. Chem. Int. Ed. Engl. 51* (2012) 11136-40.

61. T. Lukezic, U. Lesnik, A. Podgorsek, J. Horvat, T. Polak, M. Sala, B. Jenko, P. Raspor, P. Herron, I. Hunter, H. Petkovic, Identification of the chelocardin biosynthetic gene cluster from *Amycolatopsis sulphurea*: a platform for producing novel tetracycline antibiotics, *Microbiology, 159* (2013) 2524-32.

62. L.A. Mitscher, J.V. Juvarkar, W. Rosenbrook, Jr., W.W. Andres, J. Schenk, R.S. Egan, Structure of chelocardin, a novel tetracycline antibiotic, *J. Am. Chem. Soc. 92* (1970) 6070-1.

63. R.J. Theriault, L. Guagliardi, P.B. Hudson, L.A. Mitscher, Y.H. Park, J.K. Swayze, Microbiological conversion of 4-epi-cetocycline to 2-decarboxamido-2-acetyl-4-dedimethyl-9-methyltetracycline, *J. Antibiot. (Tokyo) 35* (1982) 364-6.

64. L.A. Mitscher, J.K. Swayze, T. Hogberg, I. Khanna, G.S. Rao, R.J. Theriault, W. Kohl, C. Hanson, R. Egan, Biosynthesis of cetocycline, *J. Antibiot. (Tokyo) 36* (1983) 1405-7.

65. U. Lesnik, Kloniranje genov za biosintezo tetraciklinov iz izbranih bakterij redu *Actinomycetales*, *PhD Thesis,* University of Ljubljana, Slovenia (2009).

66. H. Petkovic, P. Raspor, U. Lesnik, Genes for biosynthesis of tetracycline compounds and uses thereof. *US Patent 8361777* (2013).

67. N. Menendez, M. Nur-e-Alam, A.F. Brana, J. Rohr, J.A. Salas, C. Mendez, Biosynthesis of the antitumor chromomycin A3 in *Streptomyces griseus*: analysis of the gene cluster and rational design of novel chromomycin analogs, *Chem. Biol. 11* (2004) 21-32.

Mathematical Modelling of Virus Replication Kinetics as a Function of Biological Reaction System Properties

Marijan Bošnjak[1*], Daslav Hranueli[2], Jo Maertens[3], Davor Valinger[2], Želimir Kurtanjek[2] and Erick J. Vandamme[3] *Original Scientific Paper*

[1]Croatian Academy of Engineering, Kačićeva 28, 10000 Zagreb, Croatia

[2]Faculty of Food Technology and Biotechnology, University of Zagreb, Pierottijeva 6, 10000 Zagreb, Croatia

[3]Faculty of Bioscience Engineering, Ghent University, Coupure Links 653, 9000 Gent, Belgium

The danger of damages of industrial microbial production processes caused by infections with microbial viruses, and increased frequency of pathogenic microbes resistance against different antibiotics applied in therapies of infective diseases induces growing interests of scientists in propagation studies of microbial viruses, especially of those capable to destroy pathogenic microorganisms. In the present work, the kinetics of mycovirus and actinophages propagations was studied to define propagation process relationships. Appropriate mathematical models were developed and their applicability was tested. Own already published experimental data and findings referring to processes of glucoamylase production by *Aspergillus niger* with mycovirus infected strain, and Oxytetracycline biosynthesis by *Streptomyces rimosus* infected with actinophages were used to develop these mathematical models. These were then used to perform computer simulations of mentioned processes. Experimental data were compared with the simulation results. An acceptable agreement of the model simulations and the experimental data was established. Since one of the aims of this study was to develop mathematical models which could serve as a basis for model based studies with a view to stopping infective diseases caused by pathogenic microorganisms after applying phage therapies, one could hope the further actions would result with appearance of applicable such mathematical models.

Key words:

Mathematical modelling, microbial growth, virus propagation, kinetics

[*]Corresponding author: marijan.bosnjak@hatz.hr

1. Introduction

As pointed out already at the beginning of the 2nd half of 20th century by Pelczar and Reid (*1*), viruses are alike in that they are all obligate parasites that live in the cells of their selected hosts. Actually it is known that their selected hosts can be man and living organisms belonging to different genera of plants and animals, *i.e.* selected living cells of Eukaryotes and Prokaryotes. A series of infectious diseases are caused by viruses. Already at the beginning of 20th century it was observed by Twort in England and by d'Herrelle in France (*1*) that bacteria can be infected by viruses.

In his article published recently Vandamme (*2*) explained the history and perspective of bacterial viruses (phage's) application in medicinal phage therapy. Prior to the discovery of penicillin the interest for the study and application of phage therapy in medicine was significant. However, concomitant with the increasing number of discovered antibiotics the interest for phage therapies decreased to the level of neglecting or even excluding phage therapy, except in Georgia (formerly one of the Soviet republics). However, the interest for study and application of phage therapy renews with the significantly increased frequency of the appearance of pathogenic microbes being resistant to antibiotic action. In their recent review Kutateladze and Adamia (*3*) confirmed Vandamme's statements and pointed the multiple advantages of combined phage therapies in comparison to therapies with antibiotics. In addition, one should mention that phages and other viruses became a favourite object of study for biochemists, geneticists and molecular biologists (Vandamme, 2000). In contrast to desired useful phage effects when being applied as therapeutic agents, the pointed out undesirable phage contaminations in the fermentation industry (*2*) provoked interested scientists to be engaged in studies of how to prevent such contaminations. The efforts have been successful, both with respect to preventing phage propagation in fermentation processes of a series of different industrial biotechnological production processes, and with respect to the number of published scientific papers. The latter is partly illustrated by one of these publications referring to the use of actinophages being infective against streptomycetes producing antibiotics (*4*), as well as by other publications referring to phages acting infectively against other bacteria applied in industrial microbial productions (*4-7*).

Eukaryotic microorganisms can also be infected by viruses, and there are numerous publications referring to their effects on fermentation processes applied in industry. Process relationships were studied as well. Valinger and collaborators studied the kinetics of mycovirus propagation during glucoamylase production in repeated fed batch cultures of a glucoamylase producing virus infected *A. niger* strain (*8*). Mathematical modelling was applied in order to define process relationships. An acceptable agreement between experimental and computer simulation data was obtained.

Since infections with viruses are characterised by their linking onto the outer barrier of host cells followed by the entrance of virus nucleic material into attacked cells and the multiplication of entered nucleic material based on nucleic materials of host cells, disregarding whether the host organism is unicellular or multi-cellular, one can consider the analogue approaches to mathematical modelling of virus replication kinetics in infected organisms can be applied. Therefore, all appropriate process events should be considered in order to select the most relevant ones to describe adequately process kinetics by mathematical models. In this paper, the first step in this direction is pursued by comparing own already published data with a mathematical model.

The data that will be used in this study are collected from cultures of *A. niger* with virus infection (*8*), and from cultures of *S. rimosus*, *i.e.* strains known as Oxytetracycline producers differing in their susceptibility to actinophages infections (*4* and references in *9*).

2. Mathematical modelling

Different approaches to mathematical modelling of biochemical and biological reaction systems are applied. However, concerning the kinetics of very complex processes the most convenient approaches prefer the application of mathematical models developed for computer simulations. Therefore, such an approach could be applied in describing mathematically process kinetics in reaction systems where viruses participate as process characterising factors. Prior to starting mathematical modelling one should distinguish relevant specific properties of particular reaction systems where viruses are present as active factor. This means that one should take into account whether virus infection and propagation refer to systems of cultivated dispersed individual microbial cells and/or their agglomerates, or to systems of cultivated cells and/or tissues of multicellular organisms, or to whole multicellular organisms or their specific tissues or organs, and whether host cells are in propagation phases or in some kind of stationary phase. One should also take into account whether virus particles as structural parts are incorporated in given organism cells, accompanying host cell propagation.

As generally known and already mentioned (*10*), the contacts, or even collisions, between reactants represent the main prerequisite for reaction process starts and development. In the case of virus infections and consequent propagation of viruses in infected cells one can consider the following events take place: a) contacts between virus particle reactive door and host external cell surface; b) passing of virus nucleic material through the host cell barrier into cell interior; c) transformation of

cell nucleic materials into virus nucleic material and followed formation of virus walls from host cells protein; d) disruption of host cells and releasing of the formed virus particles for new contacts with uninfected host cells.

In accordance to the planned actions mentioned in the section Introduction, the mathematical modelling of growth kinetics of *A. niger* microbial biomass and kinetics of accompanied mycovirus propagation could be performed primarily. Therefore, based on mentioned previous information (*8*) and relevant information referring to oxygen transfer relationships (*11,12*), for mycovirus propagation the following system of differential equations, the mathematical model MM1, can be applied:

Growth kinetics of mycelia microbe

Biomass growth:

$$\frac{\mathrm{d}}{\mathrm{d}t}\gamma_X = k_1 \cdot \gamma_X^{2/3} - k_2 \cdot \gamma_X - v \cdot \frac{\gamma_X}{K_v + v \cdot t} \qquad [1]$$

Substrate uptake:

$$\frac{\mathrm{d}}{\mathrm{d}t}\gamma_S = -q_S \cdot \gamma_X \cdot \frac{\gamma_S}{K_S + \gamma_S} + v \cdot \frac{\gamma_{S0} - \gamma_S}{K_v + v \cdot t} \qquad [2]$$

Oxygen transfer and uptake:

$$\frac{\mathrm{d}}{\mathrm{d}t}\gamma_O = k_L a \cdot \left(\gamma_{Om} - \gamma_O\right) - qo \cdot \gamma_X \cdot \frac{\gamma_S}{K_S + \gamma_S} + v \cdot \frac{\gamma_{Om} - \gamma_O}{K_v + v \cdot t} \qquad [3]$$

Virus propagation kinetics:

Virus propagation expressed as product formation:

$$\frac{\mathrm{d}}{\mathrm{d}t}N_{vp} = q_{vp} \cdot \gamma_X \cdot \left(N_{vpm} - N_{vp}\right) - k_d \cdot \frac{N_{vp}}{K_v + v \cdot t} \qquad [4]$$

Autonomous virus propagation:

$$\frac{\mathrm{d}}{\mathrm{d}t}N_v = k_3 \cdot N_v^{2/3} - k_4 \cdot N_v - v \cdot \frac{N_v}{K_v + v \cdot t} \qquad [5]$$

Equations [1] to [5] describe process kinetics in batch, fed-batch and continuous cultures. For batch cultures the volumetric rate (v) of sterile medium addition to the culture is zero, whereas this variable in the case of fed-batch cultures has

positive value. In the case of continuous cultures v maintains constant and refers simultaneously to both sterile medium input rate to the culture and culture output rate.

Since the system of *S. rimosus* cultures subjected to actinophages infection differs from that of *A. niger* cultures, a partly different system of differential equations can be proposed to describe microbial cell growth and actinophages propagation kinetics. Based on already published experimental data (*4*) a simplified mathematical model, MM2, was formulated at the start in order to test degree of its applicability:

Growth kinetics of viable microbial cells:

$$\frac{\mathrm{d}}{\mathrm{d}t}N_x = \mu_x \cdot N_x \cdot \left(1 - \frac{N_x}{N_{xm}}\right) \qquad [6]$$

Substrate uptake kinetics:

$$\frac{\mathrm{d}}{\mathrm{d}t}\gamma_s = -q_s \cdot N_x \cdot \frac{\gamma_s}{K_s + \gamma_s} \qquad [7]$$

Actinophages production kinetics:

$$\frac{\mathrm{d}}{\mathrm{d}t}N_{pv} = k_{vp} \cdot N_x \cdot \left(N_{pvm} - N_{pv}\right) - k_{dv} \cdot N_{pv} \qquad [8]$$

Autonomous actinophages propagation kinetics:

$$\frac{\mathrm{d}}{\mathrm{d}t}N_v = \mu_v \cdot N_v \cdot \left(1 - \frac{N_v}{N_{vm}}\right) - k_{va} \cdot N_v \qquad [9]$$

However, it was reasonable to suppose that more structured model MM3 would be more adequate:

Growth kinetics of non-infected microbial cells:

$$\frac{\mathrm{d}}{\mathrm{d}t}N_{xn} = \mu_{xn} \cdot N_{xn} \cdot \frac{N_{xn}}{K_x + N_{xn}} \cdot \left(1 - \frac{N_{xn}}{N_{xm}}\right) - k_i \cdot N_{xn} \cdot N_{pv} \qquad [10]$$

Kinetics of cell-actinophages contacts:

$$\frac{\mathrm{d}}{\mathrm{d}t}N_{xa} = k_i \cdot N_{xn} \cdot N_{pv} \qquad [11]$$

Kinetics of accumulation of microbial cells with actinophages infection:

$$\frac{\mathrm{d}}{\mathrm{d}t}N_{xi} = k_b \cdot N_{xa} + \mu_{xi} \cdot N_{xi} \cdot \left(1 - \frac{N_{xi}}{N_{xm}}\right) - k_{di} \cdot N_{xi} \qquad [12]$$

Kinetics of total viable microbial cells accumulation:

$$\frac{\mathrm{d}}{\mathrm{d}t}N_x = \frac{\mathrm{d}}{\mathrm{d}t}N_{xn} + \frac{\mathrm{d}}{\mathrm{d}t}N_{xi} \qquad [13]$$

Kinetics of substrate uptake:

$$\frac{\mathrm{d}}{\mathrm{d}t}\gamma_s = -q_s \cdot N_{xn} \cdot \frac{\gamma_s}{K_s + \gamma_s} \qquad [14]$$

Kinetics of actinophages production:

$$\frac{\mathrm{d}}{\mathrm{d}t}N_{pv} = k_{vp} \cdot N_{xi} \cdot \frac{N_{xi}}{K_p + N_{xi}} \cdot \left(N_{pvm} - N_{pv}\right) - k_{\mathrm{dv}} \cdot N_{pv} - k_i \cdot N_{xn} \cdot N_{pv} \qquad [15]$$

Kinetics of actinophages propagation:

$$\frac{\mathrm{d}}{\mathrm{d}t}N_v = \mu_v \cdot N_v \cdot \left(1 - \frac{N_v}{N_{vm}}\right) - k_{va} \cdot N_v \qquad [16]$$

A more complex situation occurs if virus infection refers to multicellular organisms, *e.g.* to mammalian bodies and/or their parts, especially if these are infected by pathogenic microorganisms. Then the kinetics of microbial infection and of organism immunity development, as well as pharmacokinetics of applied drugs should also be considered and taken into account. If, additionally, the phage therapy is planned to be applied, then mathematical modeling of kinetics of all mentioned events should be performed. Hypothetical mathematical models published recently (*11*) refer to pharmacokinetics and kinetics of microbial processes *in vivo*. Based on them and on these represented by equations [1] to [16] one could model mathematically phage therapy of mammalians infected by pathogenic microorganisms. Instead of extension of the text of this work towards such a direction one can rather recommend to prepare new work. This will be addressed in the following publication.

3. Materials and methods

3.1 Experimental data source

Parts of already published experimental data (*4,5*) served for testing the convenience of mathematical models described by equations [1] to [16].

3.2 Computer simulations

Due to the series of previous successful applications, especially of those recent (*10-14*) of Scientist computer programme (Micromath, St. Louis, MO, USA), it was also applied in this work. Fittings of computer simulation to experimental data were statistically validated applying the Jacobian matrix, installed as part of Scientist calculation programme. It should be noted that kinetics of substrate and oxygen uptakes have not been specifically elaborated in this work disregarding the fact of their mathematical description in presented mathematical models, since the generally relevant data can be found in already published documents (*8* and *10-14*). Applicability of equations [2] and [3] was proven (*11,12*).

The model is evaluated for its "information content" evaluated by the normalized Akaike Information Criterion (AIC), here termed as the Model Selection Criterion (MSC), defined by the formula:

$$MSC = \ln\left(\frac{\sum_{i=1}^{n}\left(y_{\mathrm{exp},i} - \overline{y}_{\mathrm{exp}}\right)^2}{\sum_{i=1}^{n}\left(y_{\mathrm{exp},i} - y_{\mathrm{mod},i}\right)^2}\right) - \frac{2\cdot p}{n} \qquad [17]$$

where n is the number of experimental data (samples) and p is the number of parameters in the selected model. The criterion relates the relative dispersions of the experimental data and the model residuals with account by "penalization" for model over-parameterization. Maximization of the MSC is sought as the objective for a model quality.

4. Results and Discussion

Results of computer simulations are presented in Figs. 1 to 20 and in Tables 4, 5 and 7. Figs. 1 to 3 refer to *A. niger* cultures. Parameter values with respect to them are presented in Table 1.

Table 1 – Parameter values with respect to presented figures Fig. 1 to Fig.

Figure	Parameters								
	k_1	k_2	v	k_v	q_{vp}	N_{vpm}	k_d	k_3	k_4
Fig. 1	0.126	0.0420	0.0	1.0	0.00105	9000	0.00008	1.3671	0.0660
Fig. 2	0.600	0.225	0.0	1.0	0.00085	9000	0.00008	3.420	0.190
Fig. 3	0.600	0.225	0.833	20.0	0.0028	6100	0.00008	3.420	0.190

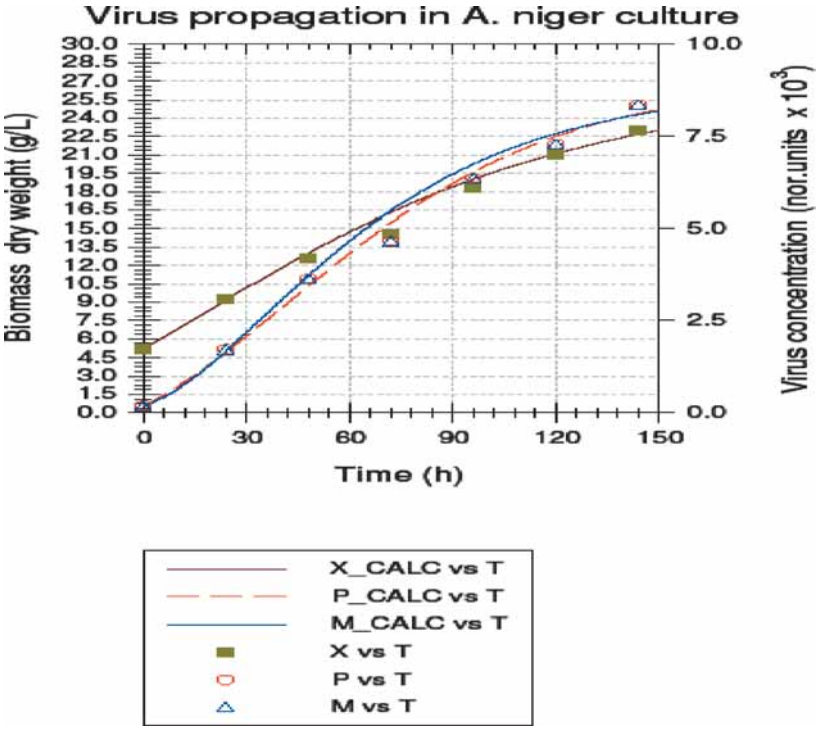


Fig. 1 – Microbial biomass growth and mycovirus propagation in function of time. Experimental data and model simulation based on MM1 (X = biomass, P = virus (as product), M = virus)

In Fig.1, one can well distinguish the differences between microbial biomass growth and mycovirus propagation. In addition, based on the evident good agreement between experimental and computer simulation data for applied parameters (Table 1), one can conclude that supposed process events relationships can be accepted. Evidently, mycovirus propagation kinetics can be explained by both relationships, that analogous to mycelia biomass growth kinetics and that corresponding to product formation kinetics. Fig. 2 confirms the findings referring to Fig. 1. Data in Table 4 demonstrate the excellent agreement between experimental and theoretical data with reference to both Fig. 1 and Fig. 2. Fig. 2 is based on the part of estimated experimental values from graphically presented data in already mentioned publication (*8*). Data of the same publication also served for process simulations of the repeated fed-batch culture. Computer simulation data presented in Fig. 3 correspond to those of the one cycle of the repeated fed-batch culture, and they are also based on the same publication (*8*). Concerning the findings presented by Fig. 1 and Fig. 2, one should point out the following: Since mycovirus is continuously incorporated in *A. niger* cells, the fact that virus replication kinetics can be well expressed by both applied relationships is quite in accordance with expectation.

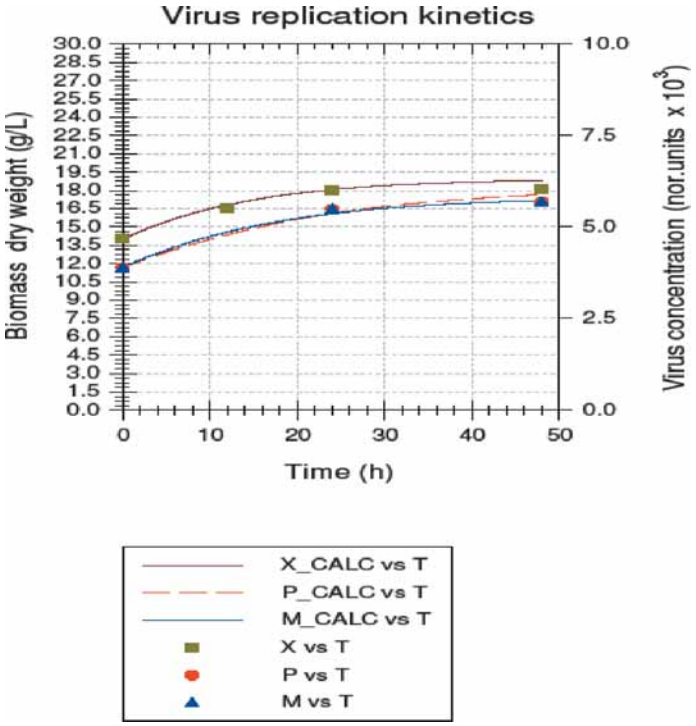


Fig. 2 – Microbial biomass growth and mycovirus propagation in function of time. Experimental data and model simulation based on MM1

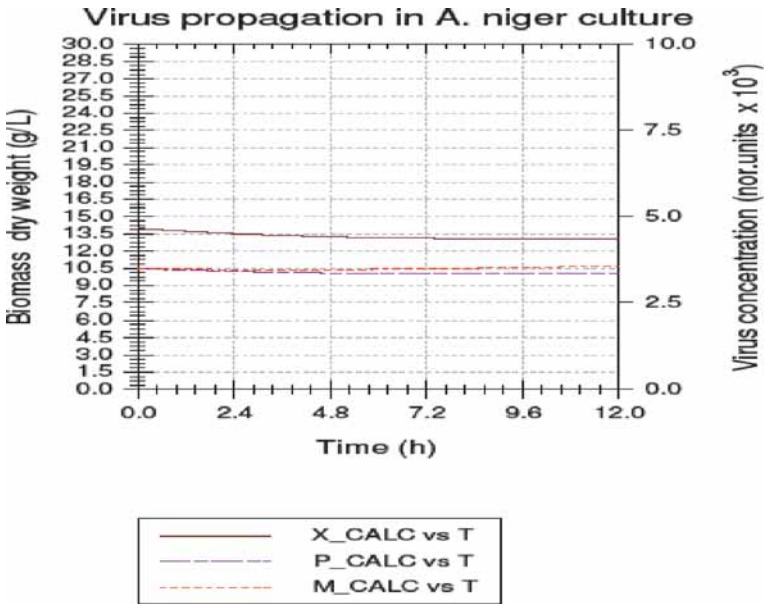


Fig. 3 – Kinetics of microbial biomass growth and mycovirus propagation during fed-batch culture. Mathematical model MM1 used in performing process simulation

In contrast to the considered relative simple process of mycovirus propagation, the process of actinophages propagation in cultures of *S. rimosus* strains appeared to be more complex for defining the process kinetics relationships.

Table 2 – Parameter values with respect to presented figures Fig. 4 and Fig. 5

Figure	Parameters									
	μx	**Nxm**	**kvp**	**Npvm**	**kdvp**	**μv**	**Nvm**	**kdv**	**Kx**	**Kp**
Fig. 4	0.16	7.0E9	1.0E–11	7.2E5	4.0E–4	0.22	8.0E5	4.5E–4	0.0	0.0
Fig. 5	0.18	7.0E9	1.3E–11	9.0E5	3.0E–4	0.22	8.0E5	4.5E–4	1.0E6	1.0E10

Such an impression resulted based on the insight into Figs. 4 and 5. Although one could consider that computer simulation data for applied parameter values (Table 2) fit enough well to experimental data when applying equations [6] and [9] for

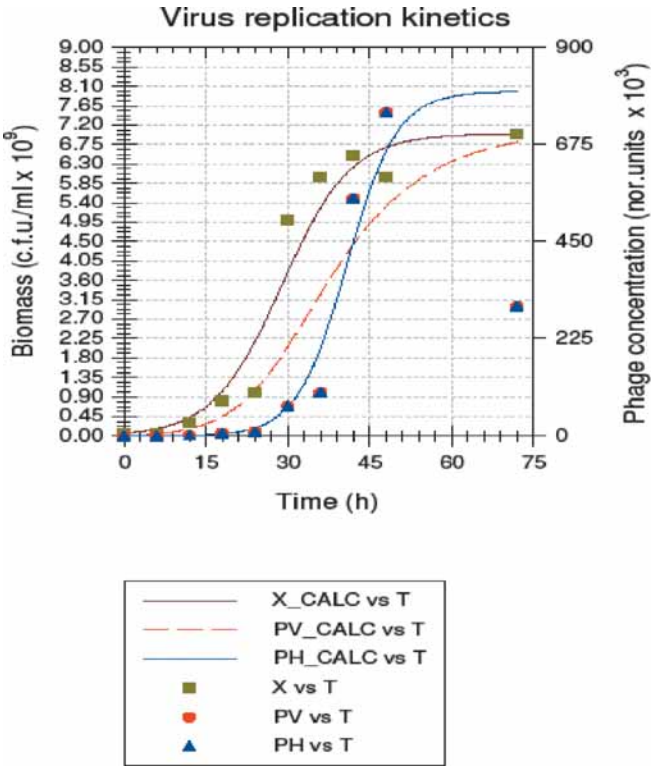


Fig. 4 – S. rimosus growth and actinophages propagation in function of time. Model simulation based on MM2 and experimental data. Remark: The last experimental actinophages concentration was neglected in statistical calculations.
(X = biomass, PV = virus(phage) as product, PH = phage)

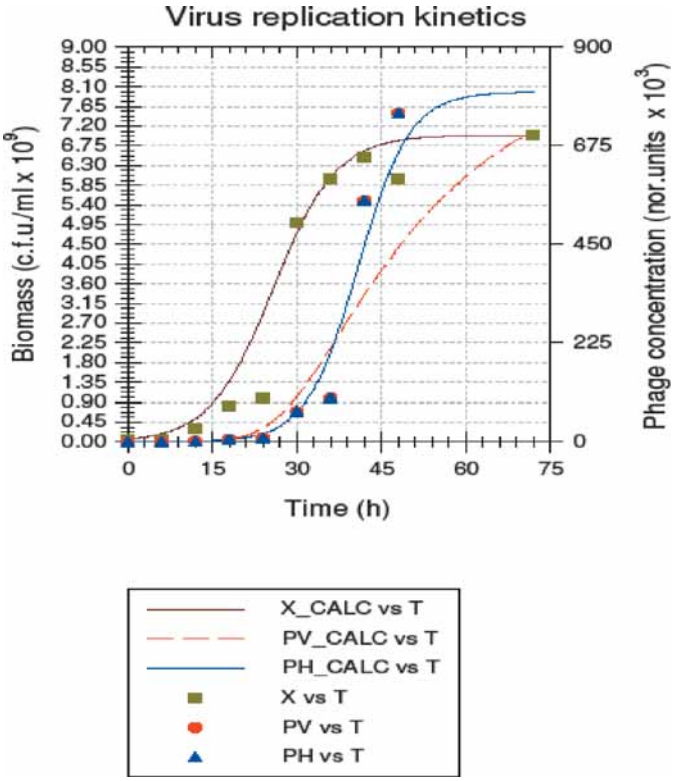


Fig. 5 – S. rimosus growth and actinophages propagation in function of time. Model simulation based on MM2 and experimental data

describing kinetics of microbial biomass growth and actinophages replication, the relationship expressed by equation [8] did not showed to be quite adequate for describing kinetics of actinophages replication. This observation suggested a modification of mathematical model and an adaptation of parameter values (Table 3) taking into account the kinetics of microbial cell infections with actinophages. Consequently, mathematical model MM3 represented by the equations [10] to [16] was formulated. Its application led to results shown in Figs. 6 and 7.

Since streptomycetes under corresponding culture conditions could be induced to form agglomerates (pellets), the relationships of cubic growth kinetics could also be applied (*11,12*), *i.e.* relationships as those for mycelia fungi. Therefore, it appeared desirable to see whether such relationship could be applicable to describe actinophages replication kinetics. Consequently, the applicability of equation [18] was investigated (MM4):

$$\frac{\mathrm{d}}{\mathrm{d}t}N_{VC} = k_3 \cdot N_{VC}^{2/3} \cdot \frac{N_{VC}}{K_z + N_{VC}} - k_4 \cdot N_{VC} \qquad [18]$$

Table 3 – Parameter values with respect to presented figures:
Fig. 6 and 7 (A); Fig. 8 to Fig. 11 (B) as well as Fig. 6, 7 and 12 (C)

A

Figure	Parameters													
	μ_{xn}	K_x	N_{xm}	k_i	k_b	μ_{xi}	k_{di}	k_{vp}	K_p	N_{pvm}	k_{dvp}	μ_v	N_{vm}	k_{dv}
Fig. 6	0.165	1.0E2	7.0E9	1.0E–6	1.0E–3	0.22	1.0E–6	3.0E–7	3.0E7	7.5E5	1.0E–6	0.225	7.5E5	3.3E–5
Fig. 7	0.165	1.2E2	7.5E9	1.0E–6	1.0E–3	0.22	1.0E–6	3.5E–7	4.3E7	7.5E5	1.0E–6	0.225	7.8E5	3.3E–5

B

Figure	Parameter values as for Fig. 7 with added the following:	k_3	K_z	k_4
Fig. 8		5.2	700.0	6.0E–5
Fig. 9		7.6	700.0	0.05
Fig. 10		16.1	1300.0	0.178
Fig. 11		19.5	1720.0	0.207

C

Figure	Parameters													
	μ_{xn}	K_x	N_{xm}	k_i	k_b	μ_{xi}	k_{di}	k_{vp}	K_p	N_{pvm}	k_{dvp}	μ_v	N_{vm}	k_{dv}
Fig. 6	0.165	1.0E2	7.0E9	1.0E–6	1.0E–3	0.22	1.0E–6	3.0E–7	3.0E7	7.5E5	1.0E–6	0.225	7.5E5	3.3E–5
Fig. 7	0.165	1.2E2	7.5E9	1.0E–6	1.0E–3	0.22	1.0E–6	3.5E–7	4.3E7	7.5E5	1.0E–6	0.225	7.8E5	3.3E–5
Fig. 12	0.165	1.2E2	7.5E9	1.0E–6	1.0E–3	0.22	1.0E–6	3.5E–7	4.3E7	7.5E5	1.0E–5	0.225	7.8E5	3.3E–5
Fig. 12	w = 22.5; z = 0.003; Nxncr = 1.2E5; Nxcrv = 1.1E5													

Data shown in Table 4 and Table 5 testify the statistically validated fitting quality of experimental with simulated data. As shown in Table 5, evidently cubic growth kinetics relationships showed to be less convenient when compared with the other two modeling approaches applied. However, an insight into Fig. 8 suggests that the cubic growth kinetics relationship can be applied during the first cultivation process phase. Fig. 11 demonstrates that the fitting of computer simulation to experimental data with reference to cubic growth relationship can be slightly improved by adapting of the computer simulation parameters (see Materials and methods).

However, data suggest that the other two relationships appeared as more convenient to describe real events. Applied positive values of K_x, K_p and K_z constants testify that process lag phases exist. A better convenience of applied the first two relationships could be considered as quite expected. Since in cultures of *S. rimosus* strains commonly the first growth phases are characterized with formation of small

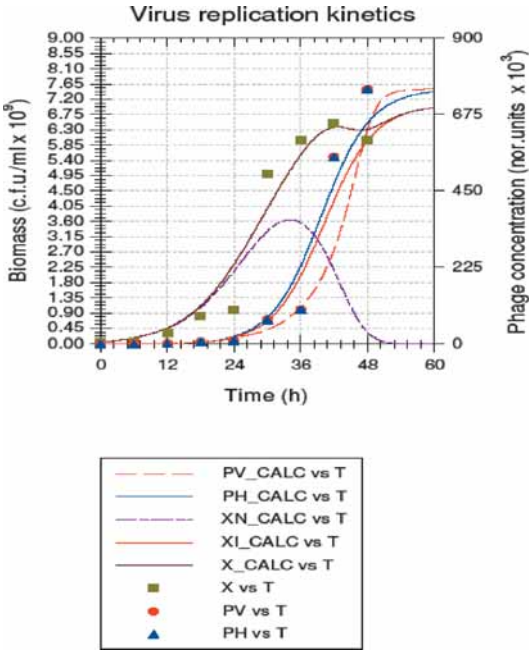


Fig. 6 – *S. rimosus* growth and actinophages propagation in function of time. Model simulation based on MM3 and experimental data

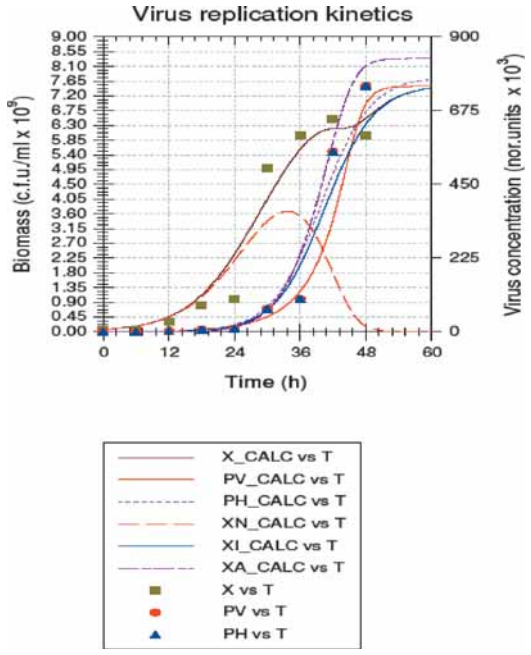


Fig. 7 – *S. rimosus* growth and actinophages replication in function of time. Model simulation based on MM3 and experimental data

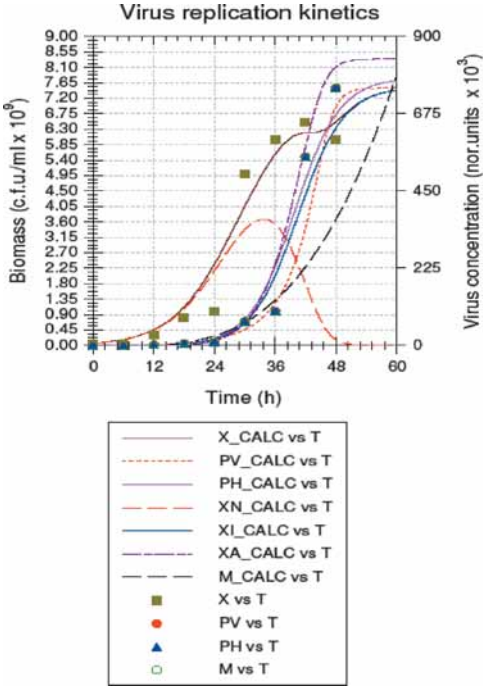


Fig. 8 – *S. rimosus* growth and actinophages replication in function of time. Model simulation based on MM4 and experimental data (letter M in simulation refers to phages (propagating cubically)

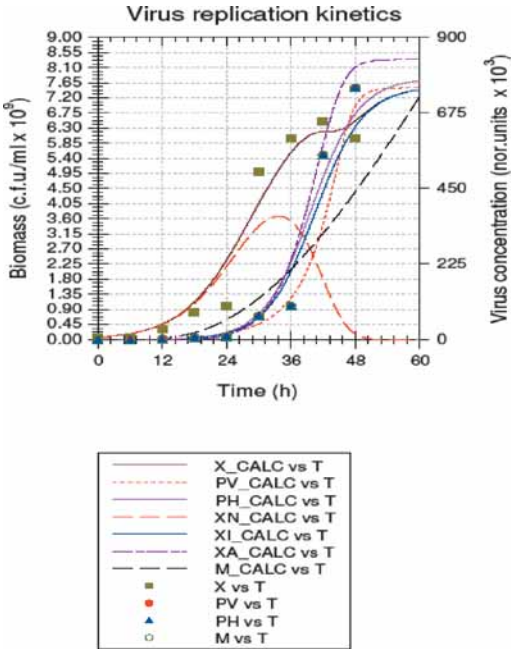


Fig. 9 – *S. rimosus* growth and actinophages replication in function of time. Model simulation based on MM4 and experimental data

pellets, the convenience of cubic growth relationship application for early growth phase could also be considered as quite expected. Probably one should point out the fact that statistically neglected the last very low experimental actinophages concentration appeared after the beginning of the phase of non-infected microbial cells exhaustion. This could mean an establishing of culture conditions inconvenient for actinophages survival and replication, and therefore convenient for enhanced actinophages concentration decay rate. Since undoubtedly microbial cell growth, cell physiology changes, cell infection with microbial viruses, cell destruction, viruses propagation and decay, are interconnected process events, one can consider the observed findings to be quite expected and scientifically acceptable. For explaining better the relatively low PV and PH value (N_{pv} and N_v number) at the end of batch culture, a transformation of the mathematical model MM3 by taking into account the effects of actinophages non-infected microbial cells showed to be necessary. As a consequence, an extending of equations [15] and [16] into equations:

$$\frac{\mathrm{d}N_{pv}}{\mathrm{d}t}=k_{vp}\cdot N_{xi}\cdot\frac{N_{xi}}{K_p+N_{xi}}\cdot\left(N_{pvm}-N_{pv}\right)-k_{dv}\cdot N_{pv}-k_i\cdot N_{xn}\cdot N_{pv}-w\cdot N_{pv}\cdot\frac{N_{xnrc}+N_{xn}}{N_{xn}} \quad [19]$$

and

$$\frac{\mathrm{d}N_v}{\mathrm{d}t}=\mu_v\cdot N_v\cdot\left(1-\frac{N_v}{N_{vm}}\right)-k_{va}\cdot N_v-z\cdot N_v\cdot\frac{N_{xncrv}+N_{xn}}{N_{xn}} \quad [20]$$

as part of new mathematical model MM5, resulted.

An application of computer simulation for parameter values w =22.5, z =0.003, N_{xncr} =1.2E5 and N_{xncrv} =1.1E5 led to the plot shown in Fig.12. Impression is that the applied mathematical model MM5 can be accepted because of satisfactory agreement of simulation and experimental data. Therefore, one can conclude that a presence of non-infected viable microbial cells is a prerequisite of viability of actinophages infected microbial cells maintenance. Due to an exhaustion of viable non-infected microbial cells the viable cells substrate for actinophages replication becomes reduced in favour to actinophages decay rate.

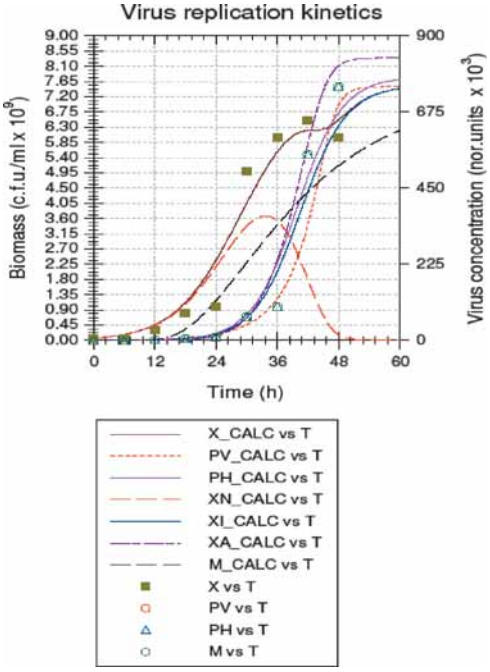


Fig. 10 – *S. rimosus* growth and actinophages replication in function of time. Model simulation based on MM4 and experimental data

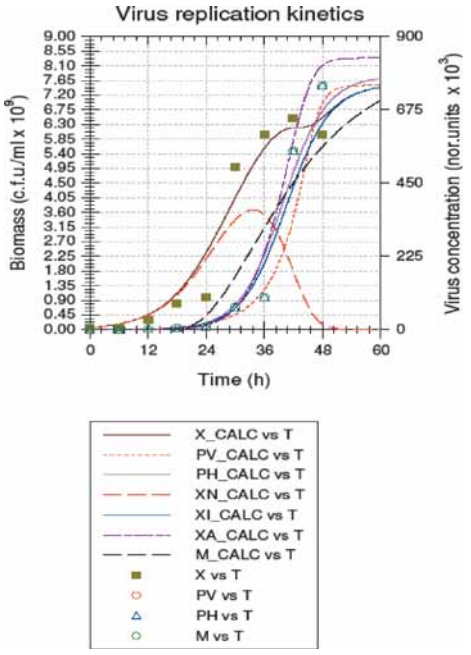


Fig. 11 – *S. rimosus* growth and actinophages replication in function of time. Model simulation based on MM4 and experimental data

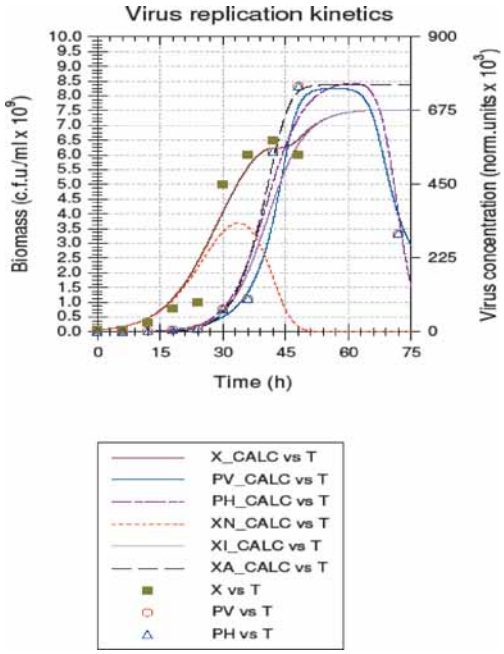


Fig. 12 – *S. rimosus* growth and actinophages replication in function of time. Model simulation based on MM5 and experimental data

Table 4 – Agreement of computer simulation and experimental data evaluated through the application of Jacobian matrix algorithm installed as one of sub-programmes in "Scientist program" (***MicroMath Scientist Statistics Report***)

Data referring to data set of the figure	Correlation	Determination coefficient	Model selection criterion
Fig. 1	0.997118639	0.993150617	3.65026331
Fig. 2	0.999461495	0.998919215	4.03006808
Fig. 4	0.986929298	0.973636596	2.92149273
Fig. 5	0.986421825	0.967537572	2.57052906
Fig. 6	0.986244090	0.972096554	2.39381988
Fig. 7	0.985239861	0.970234635	2.32922462
Fig. 8	0.985926588	0.971723333	2.51016274
Fig. 9	0.985926682	0.971723334	2.51016276
Fig. 11	0.985926782	0.971723334	2.51016278
Fig. 12	0.985527570	0.970886788	2.29518388

Table 5 – Statistical evaluation of the convenience of applied mathematical models in expressing actinophages propagation kinetics (Evaluation programme as indicated in **Table 4**; * referring to all experimental points)

Figure	Variable name	Correlation	Determination coefficient
Fig. 4	N_x, normalized microbial cell number N_{pv}, phage particle number * N_v, phage particle number *	0.976381661 0.905114738 0.791342024 0.986039294 0.830511728	0.951716464 0.778220679 0.560348965 0.953230010 0.573483942
Fig. 5	N_x, normalized microbial cell number N_{pv}, phage particle number N_v, phage particle number	0.978039348 0.952039102 0.986039294	0.940546342 0.753645269 0.953230010
Fig. 6	N_x, normalized microbial cell number N_{pv}, phage particle number N_v, phage particle number	0.977733335 0.966043796 0.981388438	0.951792439 0.857917434 0.948106227
Fig. 7	N_x, normalized microbial cell number N_{pv}, phage particle number N_v, phage particle number	0.976046093 0.977671493 0.982390761	0.948575684 0.926169384 0.955937122
Fig. 8	N_x, normalized microbial cell number N_{pv}, phage particle number N_v, phage particle number N_{vc}, phage particle number	0.976046093 0.977671493 0.982390761 0.971112955	0.948575684 0.926169379 0.955937122 0.605162594
Fig. 9	N_x, normalized microbial cell number, N_{pv}, phage particle number N_v, phage particle number N_{vc}, phage particle number	0.976046093 0.977671493 0.982390761 0.946961270	0.948575684 0.926169379 0.955937122 0.735968534
Fig. 11	N_x, normalized microbial cell number N_{pv}, phage particle number N_v, phage particle number N_{vc}, phage particle number	0.976046093 0.977671492 0.982390761 0.929594080	0.948575684 0.926169386 0.955937122 0.840803318
Fig. 12	N_x, normalized microbial cell number N_{pv}, phage particle number * N_v, phage particle number *	0.976189757 0.831793187 0.846096906	0.948972095 0.623266399 0.629589970

Although acceptable results are obtained with MM5 when describing the studied process, the model can be reformulated in a typically microbiological manner. Since the kinetics of substrate uptake was not investigated, its relevance in forming new mathematical model was actually neglected. Therefore, the following mathematical model (MM6) is proposed:

Growth kinetics of non-infected viable microbial cells:

$$\frac{\mathrm{d}N_{xn}}{\mathrm{d}t} = \mu_{xn} \cdot N_{xn} \cdot \frac{N_{xn}}{K_x + N_{xn}} \cdot \left(1 - \frac{N_{xn}}{N_{xm}}\right) - k_i \cdot N_{xn} \cdot N_{pv} - k_{xn} \cdot N_{xn} \qquad [21]$$

Kinetics of cell-actinophages contacts:

$$\frac{\mathrm{d}N_{xa}}{\mathrm{d}t} = k_i \cdot N_{xn} \cdot N_{pv} \qquad [22]$$

Kinetics of accumulation of with actinophages infected viable microbial cells:

$$\frac{\mathrm{d}N_{xi}}{\mathrm{d}t} = k_b \cdot N_{xa} + \mu_{xi} \cdot N_{xi} \cdot \left(1 - \frac{N_{xi}}{N_{xm}}\right) - k_{di} \cdot N_{xi} - k_{xni} \cdot N_{xi} \qquad [23]$$

Kinetics of non-viable microbial cells formation and decay:

$$\frac{\mathrm{d}N_{xnv}}{\mathrm{d}t} = k_{xn} \cdot N_{xn} + k_{xni} \cdot N_{xi} - k_j \cdot N_{xnv} \qquad [24]$$

Kinetics of total viable microbial cells accumulation:

$$\frac{\mathrm{d}N_x}{\mathrm{d}t} = \frac{\mathrm{d}N_{xn}}{\mathrm{d}t} + \frac{\mathrm{d}N_{xi}}{\mathrm{d}t} \qquad [25]$$

Kinetics of viable and non-viable microbial cells accumulation:

$$\frac{\mathrm{d}N_{xt}}{\mathrm{d}t} = \frac{\mathrm{d}N_{xn}}{\mathrm{d}t} + \frac{\mathrm{d}N_{xi}}{\mathrm{d}t} + \frac{\mathrm{d}N_{xnv}}{\mathrm{d}t} \qquad [26]$$

Kinetics of actinophages production:

$$\frac{\mathrm{d}N_{pv}}{\mathrm{d}t} = k_{vp} \cdot N_{xi} \cdot \frac{N_{xi}}{K_p + N_{xi}} \cdot \left(N_{pvm} - N_{pv}\right) \cdot \frac{N_{xn}}{N_{xn} + K_{xncr}} - k_{dv} \cdot N_{pv} - k_i \cdot N_{xn} \cdot N_{pv} \qquad [27]$$

Kinetics of actinophages propagation:

$$\frac{\mathrm{d}N_v}{\mathrm{d}t} = \mu_v \cdot N_v \cdot \frac{N_v}{N_v + N_{vcr}} \cdot \left(1 - \frac{N_v}{N_{vm}}\right) \cdot \frac{N_{xn}}{N_{xn} + K_{xncr}} - k_{va} \cdot N_v \qquad [28]$$

As presented by equations [21] to [28], in the mathematical model MM6 the kinetics of non-viable microbial cells formation and disintegration (autolysis) are taken into account. Hence, MM6 evidently describes the real process events in more details and more closely to supposed reality. The computer simulations performed with MM6 and applied parameters (Table 6) confirm its advantages, as clearly demonstrated by Figs. 13 to 15 and Table 7, where corresponding statistical data are included.

Table 6 – Parameters values referring to Figs. 13 to 20

Figures	Parameters													
	μxn	Kx	Nxm	ki	kxn	kb	μxi	kdi	kxni	kj	kvp	Kp	Npvm	Kxncr
Fig. 13	0.30	4.0E7	7.5E9	1.2E–6	0.07	0.2	0.20	0.01	0.25	10.0	4.5E–7	3.0E4	7.5E5	11000
Fig. 14	0.30	4.0E7	7.5E9	1.2E–6	0.07	0.2	0.20	0.01	0.25	10.0	4.5E–7	3.0E4	7.5E5	11000
Fig. 15	0.315	4.4E7	7.5E9	1.2E–6	0.06	0.2	0.20	0.01	0.30	20.0	5.6E–7	3.0E4	7.78E5	7000
Fig. 16	0.315	4.6E7	7.5E9	1.2E–6	0.06	0.2	0.20	0.01	0.30	20.0	5.6E–7	3.0E4	7.78E5	7000
Fig. 17	0.315	4.6E7	7.5E9	1.2E–6	0.06	0.2	0.20	0.01	0.30	20.0	5.65E–7	3.0E4	7.8E5	2200
Fig. 18	0.32	6.2E7	7.5E9	1.2E–6	0.06	0.2	0.20	0.01	0.30	20.0	5.65E–7	3.0E4	7.8E5	2200
Fig. 19	0.32	6.2E7	7.5E9	1.2E–6	0.06	0.2	0.20	0.01	0.30	20.0	5.65E–7	3.0E4	7.8E5	2200
Fig. 20	0.32	6.2E7	7.5E9	1.2E–6	0.06	0.2	0.20	0.01	0.30	20.0	5.65E–7	3.0E4	7.8E5	2200

Table continuation

Figures	Parameters									
	kdv	μv	Nvcr	Nvm	kva	Nmd	kNpv	kNv	k1Npv	k2Npv
Fig. 13	4.8	0.37	110	7.8E5	5.9E–2					
Fig. 14	4.8	0.37	110	7.8E5	5.9E–2					
Fig. 15	3.5	0.404	250	8.1E5	6.3E–2					
Fig. 16	3.5	0.404	250	8.1E5	6.3E–2	7.5E10				
Fig. 17	3.3	0.4075	250	8.5E5	6.3E–2	7.5E10				
Fig. 18	3.3	0.4075	250	8.5E5	6.3E–2	7.5E10				
Fig. 19	3.3	0.4075	250	8.5E5	6.3E–2	7.5E10	47.5	62.0		
Fig. 20	3.3	0.4075	250						0.097	5.5E–4

The data presented in Table 7 testify that MM6 can explain the process events with reference to the published experimental data (*4*), despite the absence of data referring to kinetics of changes of substrate concentration and kinetics of viable to non-viable microbial cells conversion. In view of the available published data concerning the kinetics of substrate uptake during the process of antibiotic production by *Streptomyces rimosus* strains (*11, 12*) extending MM6 should not represent a problem. Roughly, kinetics of phages propagation can be well explained by mathematical model MM5, when applying given parameters values in mentioned two differential equations expressing propagation kinetics of actinophages.

Table 7 – Statistical data obtained by Jacobian matrix application referring to Figs. 13 to 20

Figure	Variable name	R-squared	Correlation	Determination coefficient	Model selection criterion
Fig. 13	Variables set	0.958512500	0.977666313	0.950491423	1.48836798
Fig. 14	Variables set	0.961796780	0.980927937	0.952890553	1.58861507
Fig. 15	Variables set	0.925532021	0.955604410	0.908171476	0.921165649
Fig. 16	Variables set	0.926748876	0.957059782	0.909672004	0.937641168
Fig. 17	Variables set	0.926771435	0.957216926	0.909699833	0.937949302
Fig. 18	Variables set	0.932858654	0.966318299	0.917206150	1.02473483
Fig. 19	Nx Npv Nv Vppv Vpvv Variables set	0.932908530 0.142003295 0.170872077 0.400427577 0.205402617 0.932908521	0.940884489 0.674553056 0.683927943 0.376034833 0.678755507 0.969132999	0.845041500 0.280956707 0.237856704 0.104860995 0.186303911 0.924324467	1.70130038
Fig. 20	Nx Npv Nv Vppv Variables set	0.932691673 0.142305480 0.170982213 0.899071647 0.932691665	0.940645821 0.673667499 0.681176816 0.931883252 0.968049574	0.844540635 0.280505556 0.237692276 0.663604807 0.921571212	1.44556422

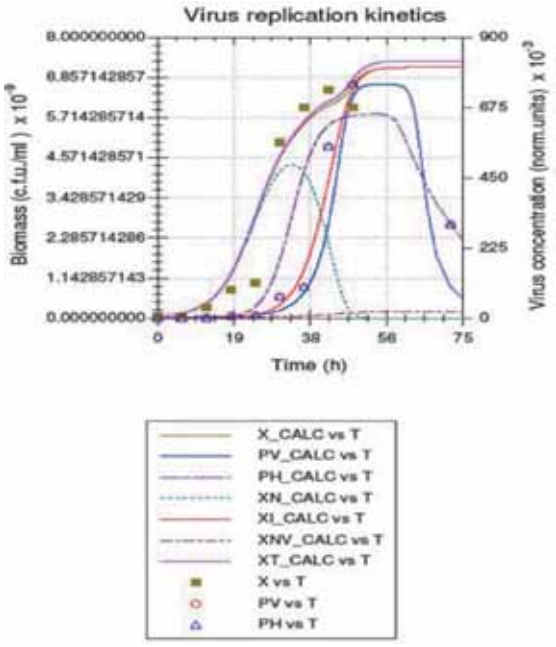


Fig. 13 – S. rimosus growth and actinophages replication as a function of time. Model simulation based on MM6 and experimental data

However, the completion of mathematical model MM6 asks for an additional equation which expresses the kinetics of accumulation of dead microbial cells, since as a consequence of loss of their viability the non-viable microbial cells succumb to processes of their death and autolysis (MM6a):

$$\frac{\mathrm{d}N_d}{\mathrm{d}t} = k_j \cdot N_{nv} \cdot \left(1 - \frac{N_d}{N_{md}}\right) \qquad [29]$$

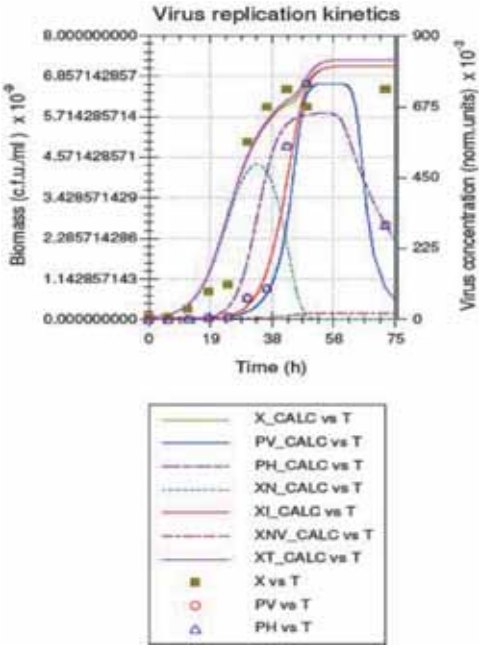


Fig. 14 – *S.rimosus* growth and actinophages replication as a function of time. Model simulation based on MM6 and experimental data

Computer simulation showed that the information given in Fig. 15 can be completed by submitting the Figs. 16 to 18. In addition the last experimental value of microbial cells concentration which was previously omitted during statistical analyses, led to improved agreement between theoretical and experimental data, after being taken into consideration. Can one define the relationship referring to value changes of the ratio between phage and microbial cells numbers, *i.e.* N_{pv}/N_x and N_v/N_x values?!

To this end, it seems to be convenient to introduce a new term named "Virus viability potential" (V_{vp}) and some hypotheses were postulated. First, the rate of its change is proportional to actual specific rate of virus concentration change. Therefore, one can try to form MM7 by extending MM6, *i.e.* by including in it the equations:

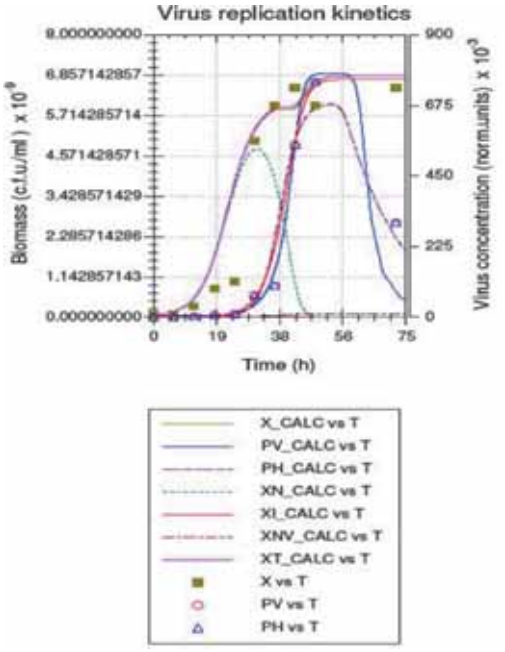


Fig. 15 – *S. rimosus* growth and actinophages replication as a function of time. Model simulation based on MM6a and experimental data

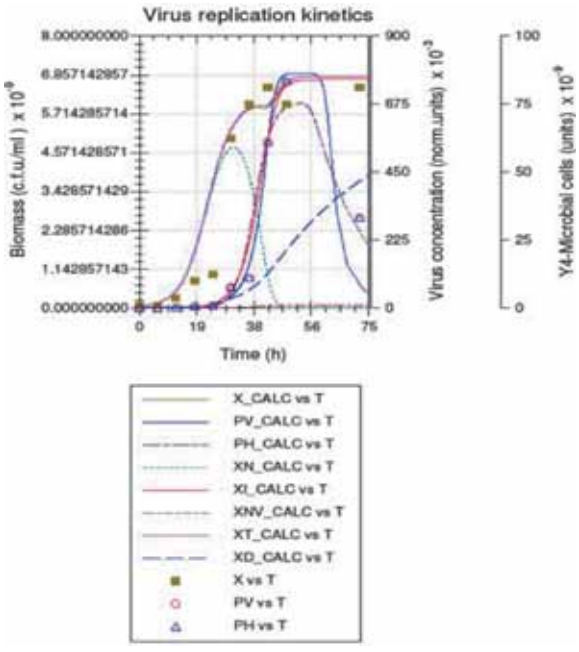


Fig. 16 – *S. rimosus* growth and actinophages replication as a function of time. Model simulation based on MM6a and experimental data

$$\frac{\mathrm{d}VN_{pv}}{\mathrm{d}t} = kN_{pv} \cdot \frac{\mathrm{d}N_{pv}}{\mathrm{d}t} \cdot \frac{1}{N_x} \qquad [30]$$

$$\frac{\mathrm{d}VN_{v}}{\mathrm{d}t} = kN_{v} \cdot \frac{\mathrm{d}N_{v}}{\mathrm{d}t} \cdot \frac{1}{N_x} \qquad [31]$$

Second, one can suppose that virus infected microbial biomass N_{xi} is relevant for expressing V_{vp}, and in accordance to such assumption to form the mathematical model MM8. Experimental data suggested that equations [30] and [31] cannot be adequate enough to express existing relationship. However, more frequent higher experimental ratio values during the first process phase suggested the applicability of following equation:

$$\frac{\mathrm{d}VN_{pv}}{\mathrm{d}t} = k_1 N_{pv} \cdot \frac{\mathrm{d}N_{pv}}{\mathrm{d}t} \cdot \frac{1}{N_{xi}} - k_2 N_{pv} \cdot \frac{\mathrm{d}N_{xnv}}{\mathrm{d}t} \cdot \frac{1}{N_{xi}} \qquad [32]$$

Namely, it is supposed that the rate of changes of V_{vp} value is the function of both specific rate of actinophages production and specific rate of nonviable microbial biomass formation, both with respect to virus infected microbial biomass. Since, as expressed by equation [28], actinophages propagation kinetics is not directly connected with kinetics of virus infected microbial biomass growth, one can exclude the probability of application of relationship analogous to equation [32] when referring to kinetics of actinophages N_v propagation. In the case of MM7 application, experimental V_{vp} values refer to ratio values calculated on the basis of both experimental N_{pv} (N_v) and N_x values, whereas in the case of MM8 application, semi-experimental V_{vp} values refer to ratio values calculated on the basis of experimental N_{pv} values and corresponding calculated N_{xi} values.

Results of computer simulations are presented in Fig. 19 and Fig. 20. Data of statistical evaluation of obtained results are presented in Table 7.

As demonstrated by Fig. 19 and Fig. 20 and supported by statistical data disclosed in Table 7, the convenience of mathematical models MM7 and MM8 certainly enlarged our insight deepness referring to cognition of real process events relationships. Fig. 19 clearly gives evidence that V_{vp} is strongly connected with participation of phage infected microbial cells in total number of viable microbial cells, and that maximal V_{vp} appeared when non-infected microbial cells mainly disappeared. Since one can consider the non-infected microbial cells represent the necessary viability substrate source for propagation of phage particles, the fall down of V_{vp} after it attained its maximal value seems to be quite expected and in accordance with expressed statement.

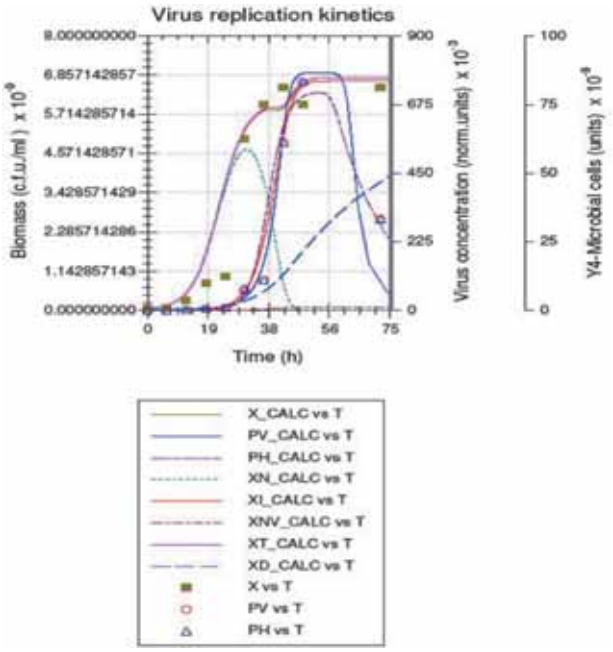


Fig. 17 – *S. rimosus* growth and actinophages replication as a function of time. Model simulation based on MM6a and experimental data

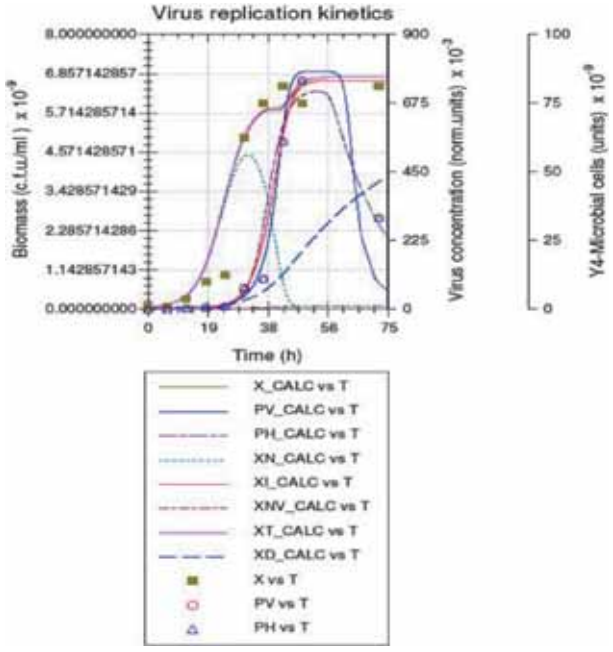


Fig. 18 – *S. rimosus* growth and actinophages replication as a function of time. Model simulation based on MM6a and experimental data

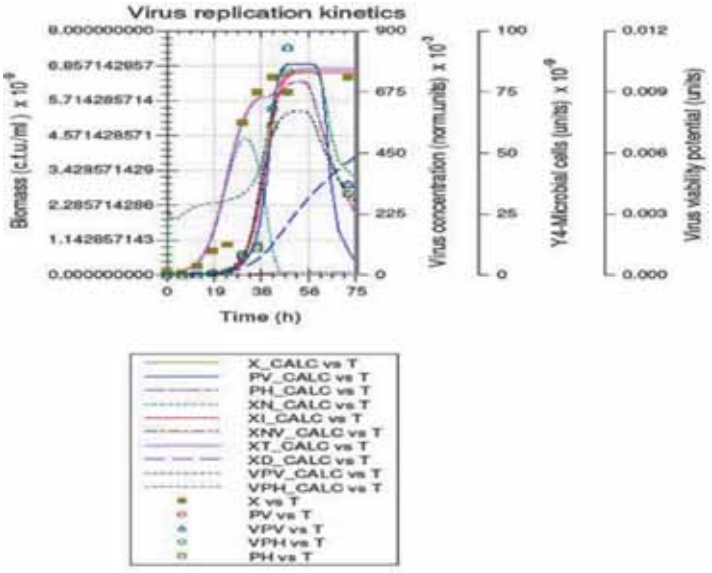


Fig. 19 – *S. rimosus* growth and actinophages replication as a function of time. Model simulation based on MM7 and experimental data

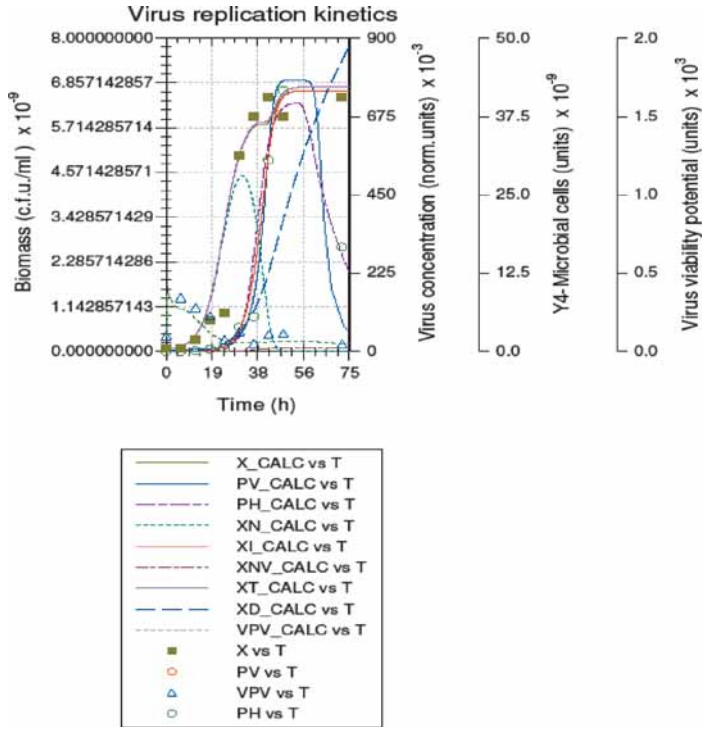


Fig. 20 – *S. rimosus* growth and actinophages replication as a function of time. Model simulation based on MM8 and experimental data

Data referring to Fig. 20 support such statement. When expressed with respect to phage infected microbial cells the higher V_{vp} values appear with evident frequency during the beginning process phase, whereas the minimal V_{vp} value appears at microbial process end.

Concerning the convenience of applied two expressions describing mathematically kinetics of actinophages propagation, one can say generally that they differed by their adequacy. Equation [27] showed as more convenient for application during the first process phase, whereas the equation [28] showed as more convenient during the second process phase. Similarly can be said comparing equation pair [30] - [31]. However, equation [32] showed to be quite adequate for expressing the kinetics of virus viability potential changes.

5. Speculative comments

In microbial batch cultures, physiological and morphological properties of microbial cells are subjected to changes. Therefore, discrepancies of experimental with respect to theoretical data can be expected. The other reason for appearance of discrepancies certainly can be due to variations of distribution of microbial cells of different physiological cell age, since as known the rates of microbial cell growth and those of grown cell divisions differ markedly. Therefore, some extent of discrepancies could be considered as being consequence of natural reproduction phenomena of microbial cells.

It is also known that phages can serve as mediators of genetic sequences transfers. Why to exclude the probability for their inducing of higher microbial cell resistance against phages entrance into microbial cell and/or for establishing of less favourable conditions in microbial cells for phages propagation, if repeated fed-batch culture experiments would be performed?!

References

1. M.J. Pelczar, jr., R. Reid: The viruses. In: Microbiology, M.J. Pelczar, jr., R. Reid (Eds.), McGraw-Hill Co. Inc., New York - Toronto - London, (1958) p.p. 156-170.
2. E.J. Vandamme, Phage therapy: viruses against bacteria, Antibiotics Today (Supplement of Chimica Oggi/Chemistry Today) (2000) 38-41.
3. M. Kutateladze, R. Adamia, Bacteriophages as potential new therapeutics to replace or supplement antibiotics, Trends in Biotechnol. 28 (2010) 591-595.
4. D. Hranueli, J. Pigac, M. Vešligaj, Characterization and persistence of actinophage RP2 isolated from *Streptomyces rimosus* ATCC 10970. J. Gen. Microbiol. 114 (1979) 295-303.

5. M. Vešligaj, M. Filipović, J. Pigac, D. Hranueli, Isolation of *Streptomyces rimosus* mutants with reduced actinophages susceptibility. Appl. Envir. Microbiol. 41 (1981) 986-991.

6. M. Welsch: Biology of actinophages. In: Symposium on Genetics and Breeding of *Streptomyces*, Dubrovnik, M. Alačević, G. Semonti (Eds.), Yug. Acad. Sci. Arts, (1969) pp. 43-62.

7. C.M. Bacq, R.W. Horne, Morphology of actinophages φ17, J. Gen. Microbiol. 32 (1963) 131-133.

8. R. Valinger, J. Beljak, M. Bošnjak, M. Ćurčić, Lj. Vitale, Kinetics of virus replication and glucoamylase biosynthesis in *Aspergillus niger* repeated fed-batch culture. Adv. Biotechnol. 1 (1981) 229-234.

9. H. Petković, J. Cullum, D. Hranueli, I.S. Hunter, N. Perić-Concha, J. Pigac, A. Thamchaipenet, D. Vujaklija, P.F. Long, Genetics of *Streptomyces rimosus*, the oxytetracycline producer. Microbiol. Mol. Biol. R., 70 (2006) 704–728.

10. M. Bošnjak, S. Bogdan, Introduction to the theoretical fundamentals for increasing the probability of heterologous contacts between biological particles, Food Technol. Biotechnol. 39 (2001) 183-195.

11. M. Bošnjak: Uvod u kinetiku mikrobnih procesa, Graphis, Zagreb, RH (2009).

12. M. Bošnjak, A. Bago Joksović, J. Pigac, Ž. Bošnjak Cihlar, D. Hranueli, Applicability of mathematical models in defining the behavior kinetics distinction among microbial strains, Chem. Biochem. Eng. Q. 20 (2006) 375-388.

13. K. Mihaljević, M. Bošnjak, Defining of the kinetics of microbial oxidation process events with reference to L-sorbose formation in a large range of culture conditions, Chem. Biochem. Eng. Q. 23 (2009) 239-250.

14. M. Bošnjak, N. Udiković Kolić, I. Petrić, D. Cihlar, D. Hršak, Integrated approach to mathematical modeling of atrazine degradation in different reaction systems, Food Technol. Biotechnol. 48 (2010) 392-403.

Symbols

a – relative surface area on the air-liquid interface (L^{-1})

d – mathematical derivation operation (dimensionless)

k – kinetic constant with reference to virus propagation decay (T^{-1})

k_1 – kinetic constant with reference to microbial biomass cubic growth ($M^{1/3}L^{-1}T^{-1}$)

k_{1Npv} – kinetic constant with reference to the specific rate of virus viability potential increase by actinophages infected microbial cells (dimensionless)

k_2 – kinetic constant with reference to microbial biomass growth decay (T^{-1})

k_{2Npv} – kinetic constant with reference to the specific rate of virus viability potential decrease by nonviable microbial cells (dimensionless)

k_3 – kinetic constant with reference to virus cubic propagation ($L^{-1}T^{-1}$)

k_4 – kinetic constant with reference to virus propagation decay (T^{-1})

k_b – kinetic constant referring to accumulation of microbial cells infected with virus

k_d – kinetic constant with reference to mycovirus propagation decay (T^{-1})

k_{di} – kinetic constant with reference to with actinophages infected viable microbial cells decay (T^{-1})

k_{dv} – kinetic constant with reference to actinophages propagation decay (T^{-1})

k_i – kinetic constant with reference to formation of microbial cell-actinophages particles (L^3T^{-1})

k_j – kinetic constant with reference to the conversion of nonviable″microbial cells into dead microbial cells (T^{-1})

k_L – linear mass transfer coefficient with reference to oxygen transfer (LT^{-1})

k_La – volumetric mass transfer coefficient with reference to oxygen transfer(T^{-1})

k_{Npv} – kinetic constant with reference to the specific rate of virus viability potential changes (dimensionless)

k_{Nv} – kinetic constant with reference to the specific rate of virus viability potential changes (dimensionless)

k_{va} – kinetic constant with reference to virus particle propagation decay (T^{-1})

k_{vp} – kinetic constant with reference to virus particle propagation (L^3T^{-1})

k_{xni} – kinetic constant with reference to the conversion of with actinophages infected microbial cells into nonviable microbial cells (T^{-1})

K_p – adaptation (lag phase) constant with reference to virus replication (L^{-3})

K_s – saturation (lag phase) constant with reference to substrate uptake (ML^{-3})

K_v – volumetric dilution (feeding) rate constant (L^3)

K_x – adaptation (lag phase) constant with reference to biomass growth (L^{-3})

K_{xncr} – constant referring to the expressed efficiency of critical non-infected microbial cells concentration (L^{-3})

K_z – adaptation (lag phase) constant with reference to virus replication (L^{-3})

N_d – concentration of dead microbial cells (L^{-3})

N_{md} – theoretically maximal concentration of dead microbial cells (L^{-3})

N_{pv} – normalized virus particles number calculated as microbial biomass product concentration (L^{-3})

N_{pvm} – theoretically maximal Npv value (L^{-3})

N_v – normalized virus particle number expressing virus concentration (L^{-3})

N_{vcr} – critical (lag phase) actinophages concentration (L^{-3})

N_{vm} – theoretically maximal Nv value (L^{-3})

N_x – normalized number of microbial cells (microbial cells concentration) (L^{-3})

N_{xa} – normalized number of particles referring to the concentration of microbial cells and virus particles in their contacts (L^{-3})

N_{xi} – normalized number of virus infected microbial cells expressing infected microbial cells concentration (L^{-3})

N_{xm} – normalized maximal microbial cell number expressing their concentration (L^{-3})

N_{xn} – normalized number of non-infected microbial cells expressing their concentration(L^{-3})

N_{xnv} – concentration of nonviable microbial cells (L^{-3})

N_{xncr} – critical normalized number of non-infected microbial cells (L^{-3})

N_{xncrv} – critical normalized number of non-infected microbial cells (L^{-3})

N_{xt} – total concentration of microbial cells (L^{-3})

q_O – specific oxygen uptake rate (kinetic constant of oxygen uptake) (T^{-1})

q_s – specific substrate uptake rate (kinetic constant of substrate uptake) (T^{-1})

t – microbial process time (T)

v – nutrient medium input flow rate with respect to microbial culture (L^3T^{-1})

V_{vp} – virus viability potential(dimensionless)

w – rate constant of actinophages production decay (T^{-1})

z – rate constant of actinophages propagation decay (T^{-1})

Greek letters

γ_O – dissolved oxygen concentration (ML^{-3})
γ_s – substrate concentration (ML^{-3})
γ_x – microbial biomass concentration (ML^{-3})
μ_v – maximal specific virus replication (propagation) rate (T^{-1})
μ_x – maximal specific microbial growth rate (T^{-1})
μ_{xi} – maximal specific growth rate referring to microbial cells infected with virus (T^{-1})
μ_{xn} – maximal specific growth rate referring to non-infected microbial cells (T^{-1})

Dimensions:

L = length, M = mass, T = time

Names of mathematical models:

MM1, MM2, MM3, MM4, MM5, MM6, MM6a, MM7, MM8

Systems Biology and Biotechnology

Želimir Kurtanjek*, Hrvoje Žilić and Ana Jurinjak Tušek *Minireview*

University of Zagreb, Faculty of Food Technology and Biotechnology
Pierottijeva 6, 10000 Zagreb, Croatia

Systems biology in present genome era receives the most important position in modern "genome based biotechnology". Its importance is result of exceptional development of experimental high-throughput techniques which deliver high dimensional multivariate data in a single biological experiment. Availabilities of large data sets are sources of biologically essential information on a system level which can be only inferred by mathematical systems theory concepts and application of mathematical/statistical methods and bioinformatics tools. The integration of information derived from a genome of industrially important microorganisms and bioprocess engineering has led to the new generation of "genome based biotechnology". Applications are very diverse, from the fundamental research in biology to applicative fields such as medicine, ecology, and most importantly in industrial biotechnology. Here is given a short review of the basic concept of the biological systems theory from a view point of biochemical engineering with the aims of white (energy production) and red (pharmaceutical production). Case studies are presented results of the system flux control analysis of *E. coli* glycolysis and the *S. cerevisiae* pathways for ergosterol and sphingolipid synthesis. Applications of the global metabolic sensitivity analysis show dynamics of adaptation (localized regulation) of *E. coli* PTS, and distributed control of glycolysis downstream metabolite (for example pyruvate). Application to the complex system of ergosterol and sphingolipid synthesis provided a dynamic "heat map" of transitions on flux control revealing the top-down structure.

Key words:
systems analysis, metabolic networks, global sensitivity, CellDesigner™

Introduction

Mathematical theories of biological systems have developed in parallel with experimental biology but initially had relatively limited impact on the science of biology. However, with the start of genome era and especially the development of many

*Corresponding author: zkurt@pbf.hr

high-throughput techniques with parallel and robot assisted systems, mathematical systems theories based on experimental molecular biology become highly developed. Most of the biological data are shared on internet in form of various open source data basis and are constantly being updated and corrected by the global scientific community. With rapid advancement of experimental techniques based on molecular tools, robotics and informatics, numerous industrially important microorganisms (about 200) have genome based (bottom-up) mathematical models of reconstructed metabolic networks.[1] In order to extract information and theories from the vast quantity of data developed are mathematical and statistical tools (chemometrics and bioinformatics) for statistical inference and data structure analysis. Simultaneously are in development mathematical theories of living systems, which in the field of biotechnology are mostly models of industrially important microorganisms as producers of energy and bulk chemicals (white biotechnology) and pharmaceuticals (red biotechnology). This biology system model approach has led to the new paradigm of "genome based biotechnology" to emphasize full potential of microorganism biological potential and a systems approach to genome engineering. The main concept is schematically presented in Fig. 1 in which a model of a producing microorganism can be approached from the "top to down" or the macro (organism) system level, or from the "bottom to up" or the molecular level. Integration and synergism of the two approaches may be the most rational road map. Although the whole field is developing at exponential rate, a fully functional and validated living cell model (living cell "in silico") on a computer is not yet available.

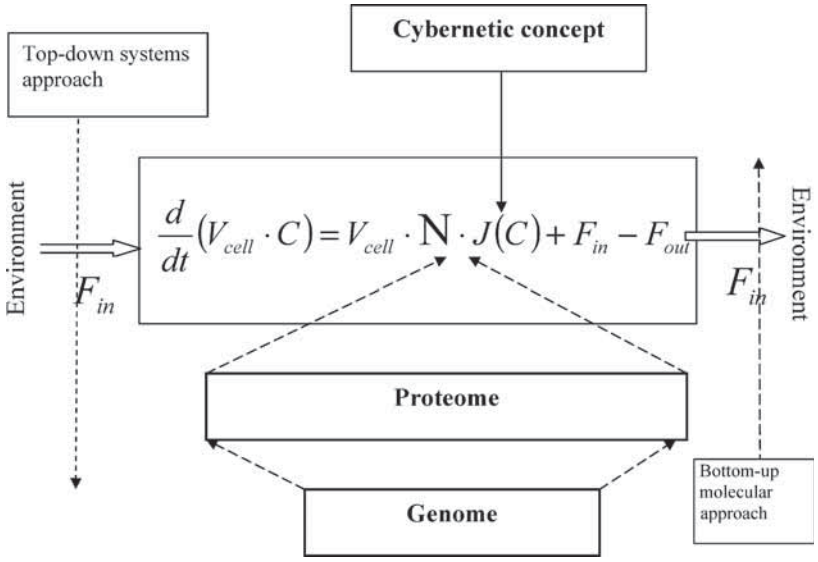


Fig. 1 – Schematic representation of the concept of "genome based biotechnology" mathematical model paradigm. N represents stoichiometric matrix of a reconstructed metabolic network, C are intracellular concentrations, F are cell input and output fluxes, and J are life goal (cybernetic) controlled intracellular fluxes (reaction rates)

For most of industrially important microorganisms (about 200) have available genomes which form the basis for development of their corresponding reconstructed metabolic networks. The knowledge of the reconstructed metabolic networks together with thermodynamic balance of free Gibbs energy for each of biotransformation reactions leads to determination of a space of feasible phenotypes.

A genome based metabolic reconstructed metabolic network are inferred from whole annotated genome, biological information and thermodynamic constraints is stored in a large metabolic stochiometric matrix *N*. Application of linear algebra in analysis of *N* provides detailed structural properties of a network topology and biological potential. Intensity of flows of energy and mass through the network are determined by thermodynamic laws but the flows are controlled based on a signaling system based on the "top to down" approach derived from the basic cybernetic principles for living systems. These two approaches are integrated into a computer model in a form of a large set of intrinsically nonlinear differential equations (Fig. 1) which represent mass balances of intracellular components connected to environment through a cell trans-membrane fluxes. Detailed analysis of these systems based models reveals new emerging properties which together with genetic engineering enables rational strategy for development of a new generation of industrial biotechnology.

Systems view cell model

Impacts of computer models and bioinformatics have dominant positions on genome based biotechnology. The central roles are genome based metabolic network reconstruction, enzyme kinetic models and multilevel regulatory mechanisms.

Dynamical transient responses of intracellular metabolite are given by:

$$\frac{1}{V}\cdot\frac{d}{dt}(V\cdot\mathbf{c})=\mathbf{N}\cdot\mathbf{J}(\mathbf{c},\mathbf{E})+\mathbf{F}_{in}-\mathbf{F}_{out} \tag{1}$$

where **N** is a stochiometric matrix of a genome level reconstructed network, **J** are intracellular fluxes, **F** are trans-membrane fluxes of molecule exchange between a cell and its environment, and **c** and **E** are metabolite and enzyme concentrations. Topological properties of the network are based on linear algebra algorithms for evaluation of potential capabilities of a specific cell. Even more importantly, is the possibility to test "in silico" various redesigning combination of a specific cellular mechanism by genetic engineering manipulation. Overall specific metabolism intensity is expressed by cell specific growth rate μ :

$$\mu = \frac{1}{V} \cdot \frac{dV}{dt} \tag{2}$$

The mass balances eq. 1 are tied up to hierarchical goal driven, but very intricate and complex, mechanism of genome transcription and regulation mechanism which could be possibly mathematically modeled on basis of systems cybernetic concepts (Fig. 1). Formally, it can be expressed by a biochemical reaction model based on the mass action principle mediated by cellular signaling network:

$$\frac{1}{V} \cdot \frac{d}{dt}(V \cdot \mathbf{E}) = \mathbf{f}(\mathbf{c}, \mathbf{E}) \tag{3}$$

The full genome scale models of the kinetic functions $\mathbf{J}(\mathbf{c}, \mathbf{E})$ and $\mathbf{f}(\mathbf{c}, \mathbf{E})$ are, at present experimental level far from reach and only fractional models for specific cellular functions have been proposed. From biochemical engineering standpoint, experimentally and theoretically possible are systems analysis based on models and data from homeostasis (chemostat) condition experiments and the corresponding FBA flux balance analysis. These models evaluate constant fluxes at different steady experimental conditions and importantly they do not involve error prone kinetic model functions and parameters.

Homeostasis systems model has algebraic structure:

$$0 = \mathbf{N} \cdot \mathbf{J} + \mathbf{F}_{in} - \mathbf{F}_{out} \tag{4}$$

Properties of the model equations, eq. 4, can be analytically fully analyzed and numerically solved, and for validated reconstructed networks are essential for genome based biotechnology.

Systems biology for white and red biotechnology

Application of systems biology for industrial application is focused on a selection and definition of a basic “chemical cell factory”. Two main candidates are yeast *S. cerevisiae* and *E. coli*. Both microorganisms are in detailed studied and applied for various productions, and mathematical models of their reconstructed networks are available. Recently, when processes of white biotechnology are considered most of research available in literature is in favor of yeast primarily due to its robustness to harsh conditions in large volume bioreactors and less environmental and health risks. When the processes of a red biotechnology are concerned, *E. coli* due to its high metabolic activity provides higher productivities of low concentration metabolites in small bioreactor volumes with strict process control. Globally, the most

important perspective of white biotechnology is production of a "second generation of biofuels", i.e. production of fuels from lower grade bio-sources (agricultural waste rather then presently used cane sugar bagasse and corn), with higher energy content per unit volume, lower emissions, better miscibility with present fuels (gasoline), lower affinity to water and corrosion[3].

The potentially main advanced biofuels are:
→ butanols (for mixture with gasoline)
→ sesquiterpentines (for jet fuel, diesel)
→ fatty acid derived biofuels

Presently is known that productivity of butanols in genetically modified *S. cerevisiae* exceeds that obtained in *E. coli*. Most of present industrial factories for production of bioethanol from corn due to the market changes resulting from surplus of hydrocarbons from shale gas will have to be retrofitted to low grade agricultural (cellulose) feed stock. The unexplored potential of synthetic biology for production of biofuels raises very expectations but is also under scrutiny of bioethical norms[2].

E. coli has been proven as a cell factory for production of various recombinant proteins. Here is presented systems analysis results of *E. coli* central metabolism with Entner-Doudoroff shunt pathway[7-8]. Analysis of the central metabolism is motivated by the fact that it is central control point for distribution of mass and energy throughout the whole metabolic network. It is also the most complex control point from which anabolic pathways spread out mostly as linear sequences of step by step transformations. Applied is the stochastic analysis of the metabolism control upon published data on glucose impulse. Control activity of each enzyme is evaluated under unsteady conditions during the first 10-15 seconds after imposed impulse under glucose deprived *E. coli* cell population in a fed-batch reactor[5]. The metabolic fluxes J are considered as a random variables defined by presumed probability distribution functions ρ for each enzyme E.

Probabilistic global parametric sensitivity

$$E\left(J^i(t)\right) = \int_{E_{1,\min}}^{E_{1,\max}} \cdots \int_{E_{N,\min}}^{E_{N,\max}} J^i\left(t, E_1, \cdots E_N\right) \cdot \rho_1\left(E_1\right) \cdots \rho_N\left(E_N\right) \cdot dE_1 \cdots dE_N \tag{5}$$

Flux control coefficients are evaluated as the global sensitivities determined by the conditional variances of the whole assembly of enzymes included into the model.

$$S_j^{Ji}(t) = \frac{\sigma^2\left(E\left(J^i(t)\middle|E_j\right)\right)}{\sigma^2\left(J^i(t)\right)} \tag{6}$$

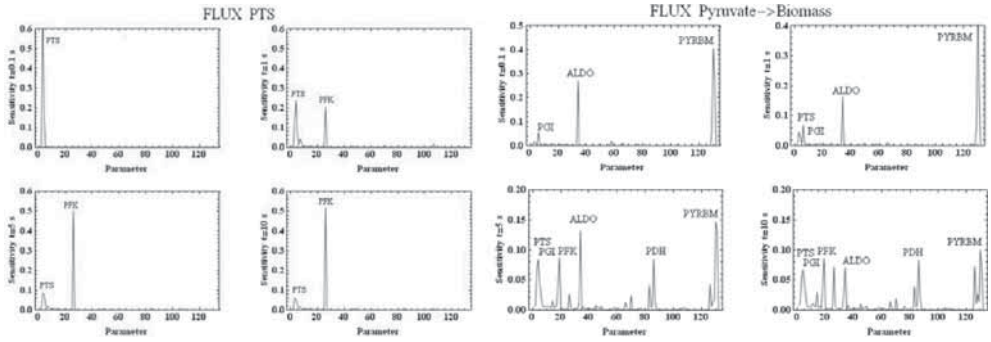


Fig. 2 – Dynamic responses of the control flux coefficients for PTS mechanism and pyruvate to biomass flux.

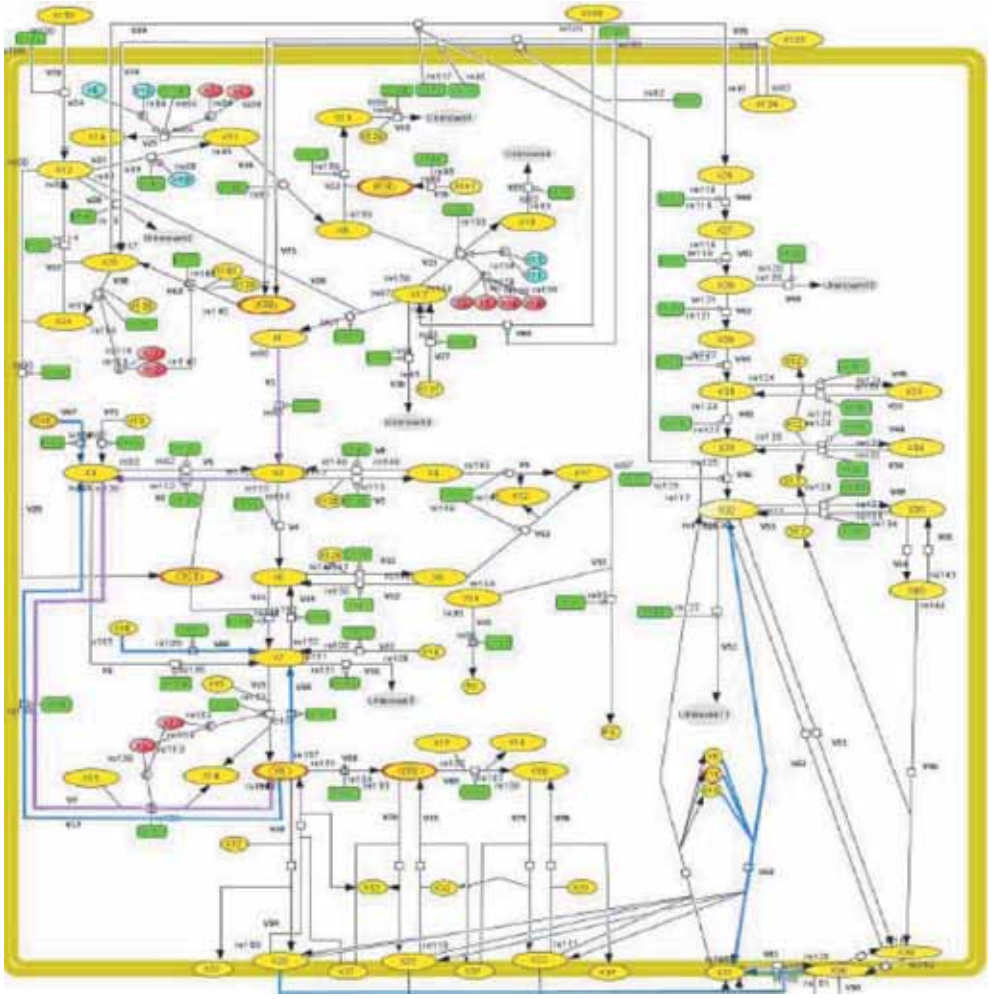


Fig. 3 – CellDesigner model of ergosterol and sphingolipds in S. cerevisiae (H. Žilić, 2013).

The computer simulations reveal the known fact that PFK enzyme is the control hub (focus) which undertakes the control from PTS enzyme complex in the very few fractions of a second. Further intake of glucose is solely controlled by PFK. However, fluxes around pyruvate and acetyl-coenzyme-A exhibit complex concerted control of multiple enzymes. These fluxes have distributed control and their redirection or amplification requires complex systems analysis.

The second example given here is the study of interaction between pathways for synthesis of ergosterol and sphingolipds in *S. cerevisiae* (H. Žilić, 2013). The model data (Alvarez-Vasquez, 2011) are given in the S-system form. The algebraic model equations are transformed to a *CellDesigner* model presented in Fig. 3 and also in Wolfram *Mathematica* software. Applied are dynamic local and global sensitivity analyses (by extended FAST method).

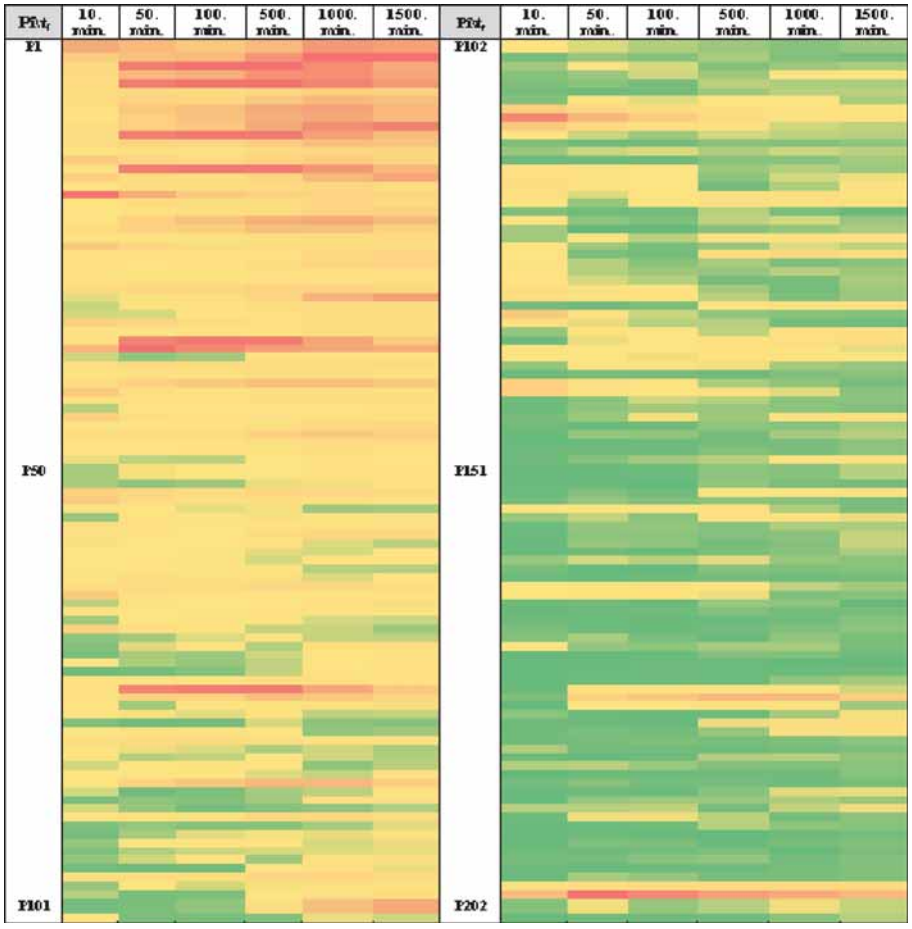


Fig. 4 – "Heat map" of dynamic flux control coefficients in the pathways for ergosterol and sphingolipds in S. cerevisiae.

Due to numerous fluxes (80) and parameters (147) the results are presented in a colored coded maps commonly called “heat maps” which are also frequently used for presentation of high-throughput experimental data. One of the obtained computer simulation scenarios is presented in Fig 4. The results indicate that there are 10 key controlling enzymes (of 80 included) which are in the initial parts of the synthesis pathways. Relatively small number of the controlling enzymes indicates the robustness of the synthesis.

Conclusions

From biochemical engineers perspective systems biology provides methodology and tools for systemic analysis of vast data available from biological experiments based on high-throughput experiments.

Importance of the systems approach is ability to extract statistically significant information and relate the biological effects to external factors tested in a controlled and designed experiments.

Systems level dynamic models provide computer simulations needed for optimization of existing technologies or design and optimization of new technologies in white and red biotechnology.

Probably the most important results of biological systems models is their use for rational planning of genetic engineering simultaneous interventions of several key enzymes and possibility for development of synthetic enzymes tailored according to computer predicted objectives.

List of symbols

c – vector of intracellular concentrations
E – enzymes
E – expected value function
F – transmembrane fluxes
f – model functions of mass balances
J – metabolic fluxes
N – stoichiometric matrix of metabolic networks
t – time
V – cell volume
σ^2 – variance
ρ – probability density function

Literature

1. Ines Thiele, Bernhard Ø. Palsson, A protocol for generating a high-quality genome scale metabolic reconstruction, *Nat. Protoc.* 2009; 5(1) 93-121.
2. Gregory E. Kaebnick, "Synthetic Biology", in Handbook of Global Bioethics (Ed. H.A.M.J. ten Have, B. Gordijin, Springer, 2014.
3. Jens Nielsen, Christer Larsson, Antonius van Maris and Jack Pronk, *Current Opinion in Biotechnology* 2013, 24:1–7
4. CellDesigner™: A modeling tool of biochemical networks, http:\\www.celldesigner.org
5. Ana Tušek, Christian Lorenz Müller, Jochen Supper, Andreas Zell, Želimir Kurtanjek, and Ivo F. Sbalzarini: "A Systems Biology Markup Language Model of a T-Cell Receptor Activated Signal Transduction Network", Proceedings of *29-th ITI 2007*
6. Degenring, D.:Dissertation. University of Rostock, 2004.
7. Ana Tušek, Ž. Kurtanjek, "Model and global sensitivity analysis of *E. coli* central metabolism", *Proceedings MATHMOD 09 Vienna*, Austria, 2009.
8. Želimir Kurtanjek, „Probabilistic Metabolic control Analysis", Systems Biology of Microorganisms, 22-24, March, 2010, Paris, France
9. Alvarez-Vasquez, F., Riezman, H., Hannun, Y.A., Voit, E.O. Mathematical Modeling and Validation of the Ergosterol Pathway in *Saccharomyces cerevisiae*. PloS ONE., 2011; 6, 1-17.
10. Hrvoje Žilić, "Mathematical parameter analysis of ergosterol biosynthesis pathways", Masters diploma, University of Zagreb, PBF, Zagreb, 2013

Selective Advantage of S-layer Surface Proteins Presence in *Lactobacillus* Probiotic Strains

Jasna Beganović*, Ksenija Uroić, Andreja Leboš Pavunc, Blaženka Kos and Jagoda Šušković *Review*

Laboratory of Antibiotic, Enzyme, Probiotic and Starter Culture Technologies, Department of Biochemical Engineering, Faculty of Food Technology and Biotechnology, University of Zagreb, Croatia

Bacterial surface (S-) S-layers are macromolecular paracrystalline arrays covering cell envelope which are composed of identical protein or glycoprotein subunits. So far, among numerous lactic acid bacteria (LAB) species these proteins have been identified only between several *Lactobacillus* members. The S-layer proteins of *Lactobacillus* strains have specific features and their biological roles have not yet been fully elucidated. Although these surface proteins are yet to be characterised, several functional roles in *Lactobacillus* cells have been proposed. In fact, some probiotic properties of *Lactobacillus* bacteria, such as adhesion, pathogens exclusion and immunomodulation have been related with the occurrence of S-layers. Hence, in this manuscript the most recent studies together with the results obtained in PhD. Jagoda Šušković, Prof. research group, concerning S-layer protein characterisation are summarised. Especially the functional role of S-layer protein in important probiotic traits, adhesion to intestinal epithelial cells, immunomodulation activity and in competitive exclusion of pathogens by *Lactobacillus* strains, is discussed.

Key words:
functionality, *Lactobacillus*, probiotic, S-layer surface proteins

Introduction

Probably the best studied lactic acid bacteria (LAB) genus is *Lactobacillus,* which is the biggest group containing over 100 species (Sengupta et al., 2013). Besides their role in food fermentations, *Lactobacillus* strains are found in the gastrointestinal tract (GIT) of humans and animals and many strains have been characterised as probiotics. These strains were reported to exert health benefits such as protection against infection by modulating the immune system. Immunomodulation and the ability to colonize mucosal surfaces have prompted efforts aimed at the use of these

*Corresponding author: jbeganov@pbf.hr

strains as vaccine delivery vehicles for oral immunization (Mobili et al., 2010). Although the molecular basis of these probiotic activities are not well understood, several mechanisms have been proposed: coaggregation with pathogens, competitive exclusion, modulation of the immune response, contribution to mucosal barrier function, decreasing of the luminal pH and secretion of specific compounds such as bacteriocins (Šušković et al., 2001; Kos et al., 2003; Frece et al., 2009; Ljungh and Wadström, 2009; Šušković et al. 2010). Still, adhesion of the probiotics to the GIT mucosa is considered a main prerequisite for their survival and establishment in the GIT where their health benefits are expected. Ability to temporarily colonize the intestinal epithelia allows probiotics to exert their beneficial effects longer. Surface-located molecules such as lipoteichoic acid, lectin-like molecules and proteins have been identified as adhesins which specifically interact with different receptor moieties in the intestinal tissue (Kleerebezem et al., 2010; Beganović et al. 2010; Hynönen and Palva, 2013). Several species of the genus *Lactobacillus* possess surface S- layer protein (SlpA). Due to their structural regularity and the unique self-assembling properties S-layers have potential for many biotechnological applications (Åvall-Jääskelä and Palva 2005; Hynönen and Palva, 2013). Although the functional significance of *Lactobacillus* S-layer proteins is not completely elucidated, these proteins are assumed to have an important role in bacteria, because a substantial part of the synthetic capacity of the bacterial cell is used for their production. The following biological functions have been shown for S-layers: protective barrier against environmental hazards, control of the transfer of nutrients and metabolites, maintenance of cell shape and cell envelope rigidity, and promoter for cell adhesion and surface recognition (Lebeer et al., 2008; Hynönen and Palva, 2013). One of the main research topics of the Laboratory of Antibiotic, Enzyme, Probiotic and Starter Cultures Technologies, Faculty of Food Technology and Biotechnology, University of Zagreb is to assess the functional role of S-layer surface proteins, in probiotic activity of different *Lactobacillus* strains isolated from autochthonous Croatian fermented products.

Probiotic activity of *Lactobacillus* strains on the host

A probiotic can be defined as a viable mono- or mixed culture of microorganisms which, when applied to animal or man, beneficially affects the host by improving the properties of the indigenous microflora (Huis in´t Veld and Havenaar, 1993; Šušković, 1996). Among LAB strains *Lactobacillus* group is the best studied species of probiotics. Numerous investigations report the functional role of probiotic strains from *Lactobacillus* genus in adhesion, which contributes to pathogen exclusion and immunomodulation (Ljungh and Wadström, 2009; Šušković et al., 2009; Šušković et al., 2010). Although lactobacilli have long been used by humans, for example in the fermentation of vegetable, dairy and meat products, in recent the ability of these bacteria to confer certain beneficial health effects has been of the

greatest interest (Bernardeau et al., 2006, Mobili et al., 2010; Leboš Pavunc et al., 2012; Beganović et al., 2013). The mechanisms underlying health-promoting capacities of the *Lactobacillus* strains belong to the one of the following, even overlapping, modes of action: i) pathogen exclusion and restoration of microbial homeostasis through microbe-microbe interactions, ii) enhancement of epithelial barrier function, iii) immunomodulation (Lebeer et al., 2008). Lactobacilli are part of the GI microbiota of humans and animals, and are also found to a lesser extent in the human genital and respiratory tracts (Zoetendal et al., 2004). As part of gut microbiota, it is estimated that *Lactobacillus* strains number 10^3-10^6/ml in the oral cavity, 10^3/ml in the stomach, and 10^4/ml in the duodenum and jejunum. The number of lactobacilli increases in the intestine with up to 10^8/ml in the ileum and 10^9/ml in colon (Walter, 2005). Somewhere in the region of $10^{7–8}$ lactobacilli are thought to be shed per gram of human faeces. Due to the relatively high numbers found there, most studies on lactobacilli in the human GIT have focused on the intestine. However, it is worth remembering that the lactobacilli here represent a relatively small proportion less than 1% of the total (Ljungh and Wadström, 2009). In all, 17 species of lactobacilli are putative inhabitants of the human gut. The most dominant are probably *Lactobacillus gasseri*, *L. crispatus*, *L. reuteri*, *L. casei*, *L. salivarus* but it is thought that some of these are transient colonizing GIT that do not remain in the host for prolonged periods of time (Walter, 2005).

Lactobacillus strains are one of the most frequently used bacterial strains as a potential probiotic. Already over a century ago Eli Metchnikoff reported that fermented milk with lactobacilli might be responsible for the increased longevity observed in Bulgarian peasants. This was the birth of the probiotic era and since then there has been a continual expansion of probiotic products available to consumers (Ljungh and Wadström, 2009). However, the requirement that the bacteria should be intact and live, may be too restrictive, as recent data suggest that bacterial constituents, such as DNA or secreted metabolites called bacteriocins also may express health benefits to the host (Isolauri et al., 2004; Šušković et al., 2010). Criteria for designating a bacterial strain as a probiotic include GRAS (generally regarded as safe) status, acid and bile stability, absence of pathogenicity, GI transit and survival, production of antimicrobial substances, and modulation of immune response (Šušković et al., 2009; Kleerebezem et al., 2010). Different strains evoke different responses in the host. Therefore, results with one specific *Lactobacillus* strain cannot be generalized. Molecular research on lactobacilli should carefully be directed to these strain-specific properties. Different probiotic *Lactobacillus* strains have been associated with different effects related to their specific capacities to express particular surface molecules, such as S-layer proteins, or to secrete proteins and metabolites directly interacting with host cells (Lebeer et al., 2008). Already in 1990, PhD Jagoda Šušković, Prof. evaluated a probiotic concept strategy during her PhD performance in Laboratory of Antibiotic, Enzyme, Probiotic and Starter Cultures Technologies. Since then in our Laboratory, numerous different LAB strains

were isolated and analysed according to complex probiotic strategy. More than 200 autochthonous strains of LAB, which have been isolated from different traditional fermented products, were identified, characterised as probiotic and/or starter cultures and deposited in the Collection of Lactic Acid Bacteria (ZBMK) in Laboratory of Antibiotic, Enzyme, Probiotic and Starter Cultures Technologies, Faculty of Food Technology and Biotechnology, University of Zagreb (Figure 1.), according to probiotic strategy established by Šušković (1996).

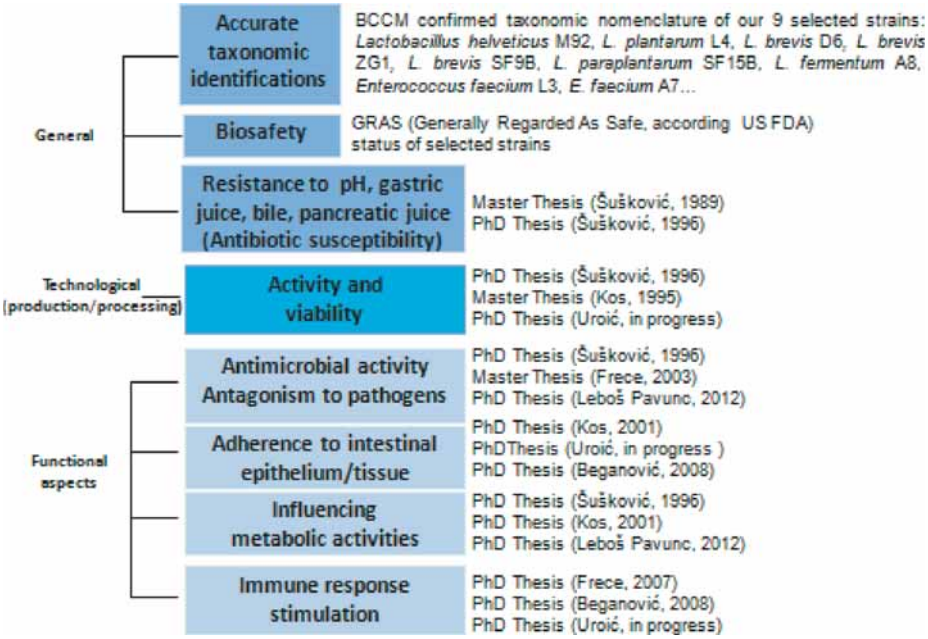


Fig. 1 – Strategy for the selection of probiotic strains in Laboratory of Antibiotic, Enzyme, Probiotic and Starter Cultures Technologies, Faculty of Food Technology and Biotechnology, University of Zagreb (adapted from Šušković et al., 2009.)

Numerous investigations report on *Lactobacillus* strain beneficial effects in the GIT and its role in conditions such as lactose intolerance, infectious diarrhoea, antibiotics associated diarrhoea, inflammatory bowel disease, irritable bowel syndrome and even in the prevention of colon cancer (Lebeer et al., 2008; Ljungh and Wadström, 2009). Later in this review, the selective advantage of S-layer surface proteins presence in *Lactobacillus* probiotic strains will be discussed.

Lactobacillus surface layer proteins

Lactobacillus strains encounter several environmental stresses during their adaptation to the host i.e. their transit through the GIT such as low pH, bile salts, and

oxidative and osmotic stress, along with starvation. Hence, *Lactobacillus* strains have developed different adaptation responses to survive these specific conditions which rely on the coordinated expression or suppression of genes that act simultaneously to improve stress tolerance (Sengupta et al., 2013). Proteins located in the cell envelope are key molecules that interface bacteria with their microenvironment (Åvall-Jääskelä and Palva, 2005). They provide various functionalities that allow bacteria to adapt to various environments, such as internalization of nutrients, export of byproducts, adhesion to biological and abiotic surfaces, and colonization of host cells (Beganović et al., 2010). To date, several lactobacillar surface proteins have been identified, which include the surface layer (S-layer) proteins; functionally diverse group of proteins anchored to the cell wall via the LPXTG motif and the so called anchorless housekeeping proteins which are classically considered cytoplasmic and whose secretion and surface-anchoring mechanisms have remained poorly known; transport proteins; as well as proteins not belonging to any of the recognized protein groups (Kleerebezem et al., 2010).

S-layers are periodic crystalline arrays that are composed of protein or glycoprotein subunits, which self-assemble to cover up to 70% of the bacterial cell surface. These proteins are noncovalently bound to the cell wall, mostly to secondary cell wall polymers: lipoteichoic acids, wall teichoic acids, and neutral polysaccharides, and assemble into surface layers with high degrees of positional order, which can cover up even completely the cell wall what is strain-depended (Lebeer et al, 2008). Several specific functions have been assigned to the S-layer from different microorganisms: protective coats, molecular sieves, molecule and ion traps, cell adhesion and surface recognition, virulence factors. These potential functions have converted the S-layers in attractive targets for biophysical studies and structural research, particularly in regard to their possible nanotechnological applications (Mobilli et al., 2010). The S-layer is not impermeable and has pores between the identical units (Hynönen and Palva, 2013). *Lactobacillus* S-layers are homopolymers of 25 to 71 kDa subunits with a relatively high isoelectric point (Åvall- Jääskeläinen and Palva, 2005). S-layer proteins represent 10-15% of the total amount of proteins in *Lactobacillus* cells, and their transcription and secretion mechanisms must be efficient and tightly regulated. Multiple promoters precede several S-layer genes of *Lactobacillus acidophilus* and *L. brevis* and are likely to ensure the efficient transcription of these genes. Also, the half-lives of mRNA encoding *Lactobacillus* S-layer proteins are relatively high, approximately 15 min, which enables efficient protein translation. The predicted lactobacillar S-layer proteins contain a conserved N- terminal signal sequence of 25-30 amino acids, which indicates that their secretion occurs via the general Sec-pathway (Hynönen and Palva, 2013). The highly efficient lactobacillar promoters regions and signal sequence have been utilized in various heterologous protein expression system, for instance, in expression of the adhesive S- layer peptides of *L. crispatus* JCM 5810 (Åvall-Jääskeläinen et al., 2002). S-layer genes and proteins have been characterized from *L. crispatus* (Sil-

lanpää et al., 2000; Antikainen et al., 2002), *L. acidophilus*, *L. gallinarum* (Ventura et al., 2002; Hagen et al., 2005), *L. helveticus* (Callegari et al., 1998; Kos et al., 2003; Frece et al., 2005a; Gatti et al., 2005; Beganović et al., 2011) and form *L. brevis* (Jakava-Viljanen et al., 2002; Åvall-Jääskeläinen and Palva, 2005; Beganović et al., 2013b), and multiple S-layer genes have been identified in the genomes of *L. acidophilus, L. amylovorus, L. gallinarum, L. crispatus, L. brevis, L. gasseri* and *L. johnsonii* (Jakava-Viljanen et al., 2002; Ventura et al., 2002). In Laboratory of Antibiotic, Enzyme, Probiotic and Starter Culture Technologies, Kos et al. (2003) revealed the presence of potential surface layer (S-layer) proteins of approximated at 45 kDa, in *Lactobacillus helveticus* M92. Further investigations were performed in order to assess the role of S-layer protein in functionality of *L. helveticus* M92, which is a model probiotic LAB in our research group (Kos et al., 2003; Frece et al., 2005a; Kos et al., 2008; Beganović et al., 2011). As in our laboratory we have isolated and established a rich collection of well characterised LAB, the strains identified as members of *Lactobacillus* species were screened for the presence of S-layer surface proteins. Commonly the presence of S- layer surface proteins are detected by SDS-PAGE. Hence, after the analysis by SDS-PAGE, only six strains revealed to express S-layer proteins were further analysed for the presence of *slp* gene by PCR with specific primers (Uroić, PhD in progress). Special focus is dedicated to the characterisation of S-layer proteins role in the probiotic activity of these strains, what will be later discussed.

Functional role of *Lactobacillus* S-layer proteins in different probiotic activities

Different research groups are investigating the role of S-layers proteins from certain *Lactobacillus* strains (Mobili et al., 2010). Considering that purified S-layers are stable toward non-physiological pH, different temperatures, some kind of proteolysis, high pressures and detergent treatments, a protective role against different stress conditions has been proposed for these protein structures. In this regard, resistance to lysozyme treatment has been related to the presence of an S-layer as the outermost cell envelope of *L. helveticus* ATCC 12046 (Lortal et al., 1992). Moreover, if S-layer is extracted with chaotropic agents such as 5M lithium chloride, microorganisms became more sensitive towards aggressive environments such as the gastrointestinal juices (Frece et al., 2005a). In this sense, the S-layers from *L. brevis* and *L. kefir* have demonstrated to efficiently coat liposomes and confer them a greater stability when exposed to bile salts, pancreatic extract, pH changes and thermal shocks (Hollmann et al., 2007), opening interesting perspectives in the development of vehicles for oral administration of drugs or vaccines, specially taking into account the GRAS status of LAB. Table 1. presents different results obtained while studying the specific probiotic activities of different *Lactobacillus* strains.

Table 1 – Overview of the results obtained while studying the specific probiotic activities of different Lactobacillus strains.

Probiotic strains	Gene	Protein	Probiotic functionality	References
L. brevis ATCC 8287	*slp*A	Adhesin	Adherence	Hyonen et al., 2002
L. helveticus M92	*slp*A	S-layer protein	Adherence, immunomodulation	Kos et al., 2003 Frece et al., 2005a
L. helveticus R0052	*slpH*	S-layer protein	Pathogen exclusion	Johnson-Henry et al., 2007
L. plantarum WCFS1	*msa*	Mannose- specific adhesin	Adherence, pathogen exclusion	Pretzer et al., 2005
L. acidophilus NCFM	*slpA*	S-layer protein	Adherence, immunomodulation	Buck et al., 2005 Konstatinov et al., 2008
L. acidophilus NCFM	*bsh*A	Bile salt hydrolase A Bile salt hydrolase B	Bile salt deconjugation, reduction of serum cholesterol	McAuliffe et al., 2005
L. amylovorus	*bsh*	Bile salt hydrolase	Bile salt deconjugation, reduction of serum cholesterol	Grill et al., 2000
L. acidophilus ATCC 4356	*slp*A	Murein hydrolase activity	Antimicrobial activity	Smit et al., 2001 Prado et al., 2008
L. crispatus ZJ001	*slp*	S-layer protein	Pathogen exclusion	Chen et al., 2007
L. plantarum NC8	*pln*A	Bacteriocin plantaricin	Antimicrobial activity	Maldonado et al., 2004
L. helveticus 481	*hlv*	Bacteriocin helveticin J	Antimicrobial activity	Joerger and Klaenhammer, 1990
L. plantarum	*pln*	plantaricin	Antimicrobial activity	Hurtado et al., 2011

In addition, S-layer would account for the probiotic properties of some strains (Beganović et al., 2011) being capable of influencing the immune response and favoring cell adhesion (Kos et al., 2003; Frece et al., 2005a; Buck et al., 2005; Jakava-Viljanen and Palva et al., 2007; Konstantinov et al., 2008). Several reports discuss the involvement of the S-layer proteins in the exclusion of enteric pathogens (Johnson-Henry et al., 2007; Prado et al., 2008). The biological functions of *Lactobacillus* S-layer proteins are poorly understood, but in some species S-layer proteins mediate bacterial adherence to host cells or extracellular matrix proteins or have protective or enzymatic functions. *Lactobacillus* S-layer proteins show potential for use as antigen carriers in live oral vaccine design because of their adhesive and immunomodulation properties and the general non-pathogenicity of the species.

Role of the S-layer proteins in adhesion of *Lactobacillus* strains

Adherence of probiotic lactobacilli to the intestinal epithelium is an important trait as it promotes gut persistence time and colonisation, interaction with host cells for the protection of epithelial cells or immunomodulation, and provides protection to the intestinal barrier by various mechanisms including antagonistic activities against pathogens (Šušković et al., 2010). *Lactobacillus* strains express a variety of adhesive surface proteins, many of which are multifunctional adhesins also involved in physiological processes in the bacteria. These adhesins can be grouped as S-layer proteins, proteins with the LPXTG surface anchoring motif, surface localized housekeeping proteins and transporter proteins. Recognized targets for lactobacillar adhesins include epithelial and phagocytic cells, extracellular matrices, mucins and circulating components. A more detailed, mechanistic knowledge of *Lactobacillus* adhesion proteins will contribute to understand their role in colonization and to develop their probiotic use (Kleerebezem et al., 2010).

Several S- layer from *Lactobacillus* strains have been identified as adhesins. Schneitz et al. (1993) observed that treatment of *L. acidophilus* cells with LiCl abolished bacterial adhesiveness to chicken intestinal cells and proposed that S-layer of *L. acidophilus* mediates the bacterial binding. Treatment of *L. kefir* and *L. parakefir* cells with LiCl, which is a routine method to release S-layer proteins from the bacterial surface, abolished the haemagglutination ability of the bacteria (Garrote et al., 2004). Similarly, treatment of *L. helveticus* M92 cells with LiCl abolished bacterial adhesiveness to mouse ileal epithelial cells (Kos et al., 2003; Frece et al. 2005a), while Chen et al. (2007) reported that depletion of S-layer of *L. crispatus* ZJ001 abolished adhesiveness to HeLa cells. Removal of S- layer proteins with LiCl may, however, simultaneously remove other cell wall proteins. Along this line, deletion of S-layer protein gene *slpA* in *L. acidophilus* NCFM abolished the bacterial adherence to a human intestinal epithelial cell line, but the authors suggested that the nonadhesive phenotype probably resulted from loss of other surface proteins bound into the S-layer (Buck et al., 2005). *L. crispatus* JCM 5810 adheres very efficiently to collagens, laminin and fibronectin, which are major components of mammalian extracellular matrices (ECM). The S- layer protein CbsA (Collagen binding S-layer protein A) extracted from *L. crispatus* JCM 5810 bacterial cell surface, bound to solubilized as well as to immobilized type IV collagen (Sillanpää et al., 2000). Inhibition studies indicated that type I and type V collagen are also recognized by CbsA. Interestingly, bacteria expressing CbsA do not bind solubilized laminin but adhere to immobilized laminin (Antikainen et al., 2002), such conformation or receptor density dependent recognition of an ECM protein has been reported for other bacteria and adhesins as well. The *cbsA* gene was cloned into *Echerichia coli*, where CbsA was expressed as a His-tagged fusion protein that exhibited collagen binding (Sillanpää et al., 2000). CbsA encodes a mature protein of 410 amino acids with typical features of lactobacillar S-layer proteins, such as a

high content of basic amino acids. CbsA has a conserved C-terminal region with pI 6.8. It is interesting that the strain *L. crispatus* JCM 5810 has another S-layer gene, termed *cbsB*, which was not expressed by *L. crispatus* JCM 5810 cells and whose recombinant form did not bind to collagens (Sillanpää et al., 2000). An extensive mutational analysis with His-tagged proteins revealed that the N-terminal region of CbsA is responsible for binding to collagens and laminin, whereas the basic C-terminus anchors the S-layer into negatively charged lipoteichoic acids on the bacterial surface (Sillanpää et al., 2000; Antikainen et al., 2002).

Treatment of *L. brevis* ATCC 8287 cell with guanidine hydrochloride (GHCl), another common method to remove S-layer from cell surface, abolished binding of this strain to Intestine 407 cells, which suggested the involvement of S-layer. Expression of fragments of the *L. brevis* S-layer protein SlpA as a genetic fusion in flagellar FliC subunits in *E. coli* conferred binding of chimeric flagella to Intestine 407 cells and to fibronectin, which confirmed the adhesive characteristics of the *L. brevis* SlpA. By testing hybrid flagella expressing different *slpA* regions, the receptor binding region in SlpA was mapped to the 81 amino acids in the N-terminal part of the protein (Hynönen et al., 2002). Analysis of antibody binding to *L. brevis* ATCC 8287 and to chimeric flagella suggested that the fibronectin binding fragment in SlpA is located on a groove inaccessible to antibodies, a reminiscent of the canyon hypothesis in viral adhesion proteins. It is interesting to note that adherence of *Lactobacillus* isolates to human intestine 407 cells has earlier been correlated with binding to multifunctional ECM protein fibronectin (Kapczynski et al., 2000; Ljungh and Wadström, 2009). The two-domain structure resembling that in CbsA has also been detected in SlpA protein of *L. acidophilus* ATCC 4356. The two S-layer proteins are related in primary sequence but adhesive functions have not been described for SlpA. A fragment containing the N-terminal two-thirds of the SlpA protein crystallized into a layer and was proposed to be composed of two subdomains with a surface exposed loop (Smit et al., 2001). The C-terminal one third of the S-layer binds to LiCl-extracted cell surface of *L. crispatus* and *Lactobacillus helveticus*, which have a closely related S-layer protein (Smit et al., 2001). The predicted amino acid sequence of *L. brevis*, *L. crispatus*, and *L. acidophilus* S-layer proteins are not identical and, hence, the two-domain structure cannot be extended to *L. brevis*. The S-layer proteins from *L. brevis* and *L. buchneri* were proposed to bind to a neutral polysaccharide moiety in the cell wall, but not to peptidoglycans or teichoic acids. It thus remains open how conserved the domain architecture and molecular interactions within the cell are in lactobacillar S-layer proteins.

Formerly, *L. johnsonii* and *L. gasseri* were regarded lacking an S-layer but, Ventura et al. (2002) identified a protein called aggregation-promoting factor in these species as an S-layer-like protein, whose amino acids composition and physical properties are similar to those in lactobacillar S-layer, which indicates that their

presence in lactobacilli is more common than presently assumed. The S-protein primary sequences are conserved only in closely related species of *Lactobacillus* (Åvall-Jääskeläinen and Palva, 2005), and the hypothesis is that variability in primary sequences, as well as in the domain architecture are related to the functional variations in lactobacillar S-layer proteins and perhaps, on a broader basis, to their functional adaptation to enhance bacterial colonization in different environments.

Role of the S-layer proteins in immunomodulation mediated by *Lactobacillus* strains

Probiotic bacteria and its components can act on the immune system and increase the body's resistance to infections, cancer and allergy by enhancing the immune response in the host (Frece et al., 2005c). Lactobacilli are able to modulate immune responses of the host by interaction with the GIT mucosa. Bacterial surfaces exhibit characteristic features known as microbe-associated molecular patterns (MAMP), which are usually cell wall components, such as lipopolysaccharides, peptidoglycan, lipoteichoic acids, and wall teichoic acids, but can also be lipids, lipoproteins, nucleic acids and proteins (Lebeer et al., 2008; Sengupta et al., 2013). Surface proteins have also been characterised as key factors involved in immunomodulation (Lebeer et al., 2008; *Šušković et al., 2010*). Namely, surface proteins from probiotic bacteria could diffuse through the mucus layer that covers the intestinal mucosa, enabling interaction with epithelial and immune cells. Sánchez et al. (2010) review about probiotic surface proteins that are involved in regulation of certain signalling pathways and cellular responses, including secretion of different effector molecules such as chemokines, cytokines or antibacterial peptides (defensins), mucus secretion, induction of changes in the surface properties, rearrangement of the tight junctions and modulation of the immune function and the response of the gut-associated lymphoid tissue (GALT) cells. Hence, when Frece et al. (2005b) revealed that after the oral immunisation of mice with viable *L. helveticus* M92 cells, the levels of serum IgA, IgG and IgM antibodies from the all groups of mice were significantly increased in comparison to the control groups of mice where levels of total IgA antibodies were shown the highest, the possible functional role of the orally administered, purified SlpA in the immunomodulation conferred by *L. helveticus* M92 in mice was studied by Beganović et al. (2011). Here it must be emphasised that *L. helveticus* M92 SlpA evoked higher total serum IgA, IgG, and IgM than *L. helveticus* M92 cells without SlpA, but the S-layer did not evoke a specific humoral immune response after oral application and as such is suitable for probiotic application as an immunomodulator. In addition, the concentrations of the serum IgA, IgG, and IgM antibodies were lower when mice were orally immunised by *L. helveticus* M92 cells without S-protein compared to the levels of antibodies determined in the samples from the group of mice orally immunised with whole *L. helveticus* M92 cells, but were still higher compared to the

control. SlpA, as the outer shell of proteins in lactobacilli, may have the highest probability of the intimate interaction with the immune cells associated with the gut (Delcour et al. 1999). Previously, between different probiotic strains assessed, *L. helveticus* M92 showed the highest capacity to activate the immune system (Frece et al. 2005b). The immunomodulation capacity of the S-layer could be one of its functions, which besides its involvement in the adhesion and certain cell surface traits such as hydrophobicity and autoaggregation of *L. helveticus* M92 contributes to the immunostimulatory activity of this probiotic bacterium. Bacterial interactions are the most accepted mechanism for the reduction of *Salmonella* count observed by *L. helveticus* M92 application, while immune responses stimulation by probiotic bacteria is also influencing on host´s defence to *Salmonella* infection (Beganović et al., 2011). Recently, Konstatinov et al. (2008) found that 45 kDa protein SlpA from the surface of *L. acidophilus* NCFM was involved in the regulation of immature dendritic cells (DC) as well as cytokine production. The cellular contacts of DCs and *L. acidophilus* NCFM involve interactions between dendritic cell-specific intercellular adhesion molecule (ICAM)-3-grabbing nonintegrin (DC-SIGN), a DC specific receptor DC-SIGN, and SlpA, the dominant protein expressed by *L. acidophilus* NCFM. Investigations whether application of the different *Lactobacillus* strains may induce the specific immune response with cross-reactive properties for pathogens in the gastrointestinal tract and if the S-layer of different *Lactobacillus* strains is responsible for immunomodulating capacity, or could it be the immunomodulator are currently underway in our laboratory (Uroić, PhD in progress).

Role of the S-layer proteins in exclusion of pathogens by *Lactobacillus* strains

The possible competitive exclusion mechanisms of probiotic action include not only direct antimicrobial activity of probiotic cells by production of antibacterial substances and by stimulation of the non-specific immune system, but also by competition for nutrients and receptors on the gut enterocytes (Kos et al., 2003; Frece et al., 2009; Šušković et al., 2010). There is some evidence that probiotics could use the same attachment site so that the pathogen is in competition for binding to the host mucosal interface and thereby could be inhibited from invading the mucosal layer. This antipathogenic mechanism is known as competitive exclusion and generally requires that the probiotic lactobacilli are administered in a preventive setup, as the displacement of a pathogen by a *Lactobacillus* strain is usually not observed. The various specific adhesins described above probably contribute to this mechanism of probiotic action, although aspecific mechanisms based on steric hindrance are also possible (Lebeer et al., 2008). S-layer proteins with adhesive properties could contribute to lactobacilli probiotic activity by the inhibition of the binding of pathogens to host tissues. This can be achieved through direct competition for attachment sites on human intestinal cells, ECM and mucus proteins, or by the block-

age of pathogen surface adhesins (Mobili et al., 2010). In addition to above, coaggregation of probiotic strains with pathogens, as well as their ability to displace pathogens through antimicrobial activity, is of importance for the therapeutic manipulation of an aberrant intestinal microbiota (Servin and Coconnier, 2003; Šušković et al., 2010). Beganović et al. (2011) reported that coaggregation was significantly reduced when *L. helveticus* M92 cells were lacking SlpA compared to the results obtained with whole *L. helveticus* M92 cells, again implicating the importance of the S-layer in this process. Coaggregation, which is thought to facilitate the clearance of pathogens during mucus flushing, is described as an additional mechanism to decrease the pathogenic load during infections. Moreover, adhesion to epithelial cells and mucus mediates colonisation of the GIT by lactobacilli and may be prerequisite for competitive exclusion of enteropathogenic bacteria and immunomodulation of the host (Perdigón et al. 2003). Johnson-Henry et al. (2007) reported that SlpA extracts from *L. helveticus* R0052 had inhibited enterohaemorrhagic *Escherichia coli* adhesion to host epithelial cells, while Buck et al. (2005) and Frece et al. (2005a) demonstrated a decrease of *L. crispatus* and *L. helveticus* M92 ability to bind to intestinal epithelial cells *in vitro* after the removal or disruption of SlpAs. Similar results were obtained for the S-layers proteins of *L. crispatus* ZJ001, which were shown to play a role in the competitive exclusion against enterohemorrhagic *E. coli* (EHEC) and *S. enterica* serovar Typhimurium (Chen et al., 2007). Another example is the putative collagen- binding protein of *L. fermentum* RC-14, which was reported to inhibit the adhesion of *Enterococcus faecalis* 1131 (Heinemann et al., 2000). For instance, it is known that S-layer protein from *Lactobacillus crispatus* is able to interact, directly, with the collagen molecules on the surface of epithelial cells (Antikainen et al., 2002). This ability could be responsible for the competitive exclusion of enteropathogens, including *E. coli* O157:H7 (Chen et al., 2007). Golowczyc et al. (2007) showed that preincubation of *Salmonella* cells with S-layer proteins from *L. kefiri* leads to changes in the surface properties of this bacterium, so that they are no longer able to invade cultured human enterocytes. That S-layer protein from *L. kefiri* is also responsible of co-aggregation with the yeast *Sacharomyces lipolitica*. For S-layer protein of *L. acidophilus* ATCC 4356 was described a murein hydrolase activity associated to the lytic activity of this protein toward the cell walls of several bacteria (Prado Acosta et al., 2008).

Conclusions

S-layers have been identified in a just a several *Lactobacillus* species. S-layers have adhesion and immunogenic functions, mediating binding of lactobacilli to the host epithelial cells and simultaneously enabling pathogen exclusion. Due to these observed adhesive and immunomodulation properties, including their high degree

of structural regularity and their self-assembly properties, the possible therapeutic applications of lactobacillar S-layers have become increasingly of interest, e.g. as targeted antigen delivery vehicles to host tissues. Taking into account all above-mentioned S-layer functions that reinforce the probiotic properties of lactobacilli, characterization of different lactobacillar S-layer proteins is key to the selection of useful strains.

Literature

1. Antikainen, J., Anton, L., Sillanpää, J., Korhonen, T.K. (2002) Domains in the S-layer proten CbsA of *Lactobacillus crispatus* involved in adherence to collagens, laminin and lipoteichoic acids and in self-assembly. *Mol. Microbiol.* 46, 381-394.
2. Åvall-Jääskelä S and Palva A (2005) *Lactobacillus* surface layers and their applications. *FEMS Microbiol. Rev.* 29, 511–529.
3. Beganović, J., Guillot, A., van de Guchte, M., Jouan, A., Gitton, C., Loux, V., Roy, K. S. Huet, H. Monod, Monnet, V. (2010) Characterization of the Insoluble Proteome of *Lactococcus lactis* by SDS-PAGE LC-MS/MS Leads to the Identification of New Markers of Adaptation of the Bacteria to the Mouse Digestive Tract. *J. Prot. Res.* 9, 677-688.
4. Beganović, J., Frece, J., Kos B, Leboš Pavunc A, Habjanič K, Šušković J (2011) Functionality of the S-layer protein from the probiotic strain *Lactobacillus helveticus* M92. *Antonie van Leeuwenhoek, Intern J General Mol Microbiol.* 100(1): 43-53.
5. Beganović J., Kos B., Leboš Pavunc, A., Uroić K., Džidara P., Šušković J. (2013a) Proteolytic activity of probiotic strain *Lactobacillus helveticus* M92, *Anaerobe*, *section Molecular biology, genetics and biotechnology* 20, 58-64
6. Beganović J., Uroić, K., Leboš Pavunc, A., Kos, B., Šušković J. (2013b) Role of S-layer proteins in probiotic activity of *Lactobacillus* strains, oral presentation, The 2nd International Symposium "VERA JOHANIDES" BIOTECHNOLOGY IN CROATIA BY 2020.
7. Bernardeau, M., Guguen, M., Vernoux, J.P. (2006) Beneficial lactobacilli in food and feed: long-term use, biodiversity and proposals for specific and realistic safety and assessments. *FEMS Microbiol. Rev.* 30, 487-513.
8. Buck, B.L., Altermann, E., Svingerud, T., Klaenhammer, T.R. (2005) Functional analysis of putative adhesion factors in *Lactobacillus acidophilus* NCFM. *Appl. Environ. Microbiol.* 71, 8344-8351.
9. Chen, X., Xu, J., Shuai, J., Chen, J., Zhang, Z., Fang, W. (2007) The S-layer proteins of *Lactobacillus crispatus* ZJ001 is responsible for competitive exclusions against *Escherichia coli* O157: H7 and *Salmonella typhimurium*. *Int. J. Food Microbiol.* 115, 307-321.
10. Delcour J, Ferain T, Deghorain M, Palumbo E, Hols P. (1999) The biosynthesis and functionality of the cell-wall of lactic acid bacteria. *Antonie Van Leeuwenhoek*. 76(1-4):159-184.
11. Frece, J., Kos B., Svetec, I. K. Zgaga, Z. Mrša, V., Šušković, J. (2005a), Importance of S-layer proteins in probiotic activity of *Lactobacilli acidophilus* M92. *J. Appl. Microbiol.* 98, 285-292.
12. Frece, J., Kos, B., Beganović, J., Vuković, S., Šušković, J. (2005b): *In vivo* testing of functional properties of three selected probiotic strain, *World J. Microbiol. Biotechnol. 21*, 1401-1408.
13. Frece, J., Šušković, J., Kos, B., Beganović, J. (2005c) Immunomodulatory Effect of Probiotic Strain *Lactobacillus acidophilus* M92 in Mice. In: *Current Studies of Biotechnology, Vol. IV:*

Biotechnology and Immuno-Modulatory Drugs, Z. Kniewald, S. Jelaska, J. Kniewald, B. Nagy, S. Novak, D. Sladić, B., Šimić, B. Vitale (Eds.), HDB/Medicinska naklada, Zagreb, Croatia pp. 153–165.

14. Frece, J., Kos, B., Svetec, I. K., Zgaga, Z., Beganović, J., Leboš, A., Šušković, J. (2009): Synbiotic effect of *Lactobacillus helveticus* M92 and prebiotics on the intestinal microflora and immune system of mice, *J. Dairy Res. 76*, 98-104.

15. Garrote, G., Delfederico, L., Bibiloni, R., Abraham, A.G., Pérez, P.F., Semorile, L., De Antoni, G. . (2004) Lactobacilli isolated from kefir grains: evidence of the presence of S-layer proteins. *J. Dairy Res.* 71, 222-230.

16. Gatti, M., Rosseti, L., Fornasari, M.E., Lazzi, C., Giraffa, G., Neviani, E. (2005) Heterogeneity of putative surface layer proteins in *Lactobacillus helveticus*. *Appl. Environ. Microbiol.* 71, 7582-7588.

17. Granato, D., Perotti, F., Massery, I., Rouvet, M., Gillard, M., Servin, A., Brassart, D. (1999) Cell surface associated lipoteichoic acid acts as an adhesion factor for attachment of *Lactobacillus johnsonni* La1 to human enterocyte like Caco 2 cells. *Appl. Environ. Microbiol.* 65, 1071-1077.

18. Golowczyc M. A., Mobili P., Garrote G. L., Abraham A. G., De Antoni G.L. (2007) Protective action of *Lactobacillus kefir* carrying S-layer protein against *Salmonella enterica* serovar *Enteritidis Int. J. Food Microbiol.* 118, 264-273

19. Grill J. P., Cayuela C., Antoine J. M., Schneider F. (2000): Effects of *Lactobacillus amylovorus* and *Bifidobacterium breve* on cholesterol. *Lett. Appl.Microbiol.* 31, 154–156.

20. Hagen, K.E., Guan, L.L. Tannock, G.W., Korver, D.R., Allison, G.E. (2005) Detection, characterization, and *in vitro* and *in vivo* expression of genes encoding S-proteins in *Lactobacillus gallinarum* strains isolated from chicken crops. *Appl. Environ. Microbiol.* 71, 6633-6643.

21. Heinemann, C., Vlieg, D. B., Janssen, H. J. Busscher, van der Mei, H. C., Reid, G. (2000) Purification and characterization of a surface binding protein from *Lactobacillus fermentum* RC-14 that inhibits adhesion of *Enterococcus faecalis* 1131. *FEMS Microbiol. Lett.* 190, 177-180.

22. Huis in't Veld, J.H.J, Havenaar, R. (1993) Selection criteria for microorganisms for probiotic use. IN: *Probiotics and Pathogenicity*, Flair No 6, J. F. Jensen, M. H. Hinton, R. W. A. W. Mulder (Edit.), DLO Spelderholt Centre for Poultry Research and Information Services, 11-19.

23. Hollmann A, Delfederico L, Glikmann G, De Antoni GL, Semorile L, Disalvo A (2007) Characterization of liposomes coated with S-layer proteins from lactobacilli. *Biochim Biophys Acta.* 1768: 393-400.

24. Hynönen, U., Westerlund Wikström, B., Plava, A., Korhnen, T. (2002) Identification by flagellum display of an epithelial cell and fibronectin binding function in the SlpA surface protein of *Lactobacillus brevis*. *J. Bacteriol.* 184, 3360-3367.

25. Hynönen U. and Palva, A. (2013) *Lactobacillus* surface layer proteins: Structure, function and applications. *Appl. Microbiol. Biotechnol.* 97 (12): S225-S243.

26. Hurtado, A., Othman, N.B., Chammem, N., Hamdi, M., Ferrer, S., Reguant C, Bordons, A, Rozès N. (2011) Characterization of *Lactobacillus* isolates from fermented olives and their bacteriocin gene profiles. *Food Microbiol*. 28 (8), 1514-1518.

27. Isolauri, E., Salminem, S., Ouwehand, A.C. (2004) Microbial gut interactions in health and disease. Probiotics. *Best Pract. Res. Clin. Gastroenterol*. 18, 299-313.

28. Jakava-Viljanen, M., Åvall-Jääskeläinen, S., Messner, P., Sleytr, U.B., Palva, A. (2002) Isolation of three new surface layer proteins genes (*slp*) from *Lactobacillus brevis* ATTC 14869 and characterization of the change in their expression under aerated and anaerobic conditions. *J. Bacteriol.* 184, 6786-6795.

29. Johnson-Henry, KC, Hagen, KE, Gordonpour, M, Tompkins, TA, Sherman, PM (2007) Surface-layer protein extracts from *Lactobacillus helveticus* inhibit enterohaemorrhagic *Escherichia coli* O157:H7 adhesion to epithelial cells. *Cell Microbiol* 9(2):356-367

30. Joerger M C and Klaenhammer T R (1990) Cloning, expression, and nucleotide sequence of the *Lactobacillus helveticus* 481 gene encoding the bacteriocin helveticin J. *J. Bacteriol.*172 (11), 6339–6347.

31. Kapczynski, D.R., Meinersmann, R.J., Lee, M.D. (2000), Adherence of *Lactobacillus* to intestinal 407 cells in culture correlates with fibronectin binding. *Curr. Microbiol.* 41, 136-141.

32. Kleerebezem, M., Hols, P., Bernard, E., Rolain, T., Zhou, M., Siezen, R.J., Bron, P.A. (2010), The extracellular biology of the lactobacilli, *FEMS Microbiol Rev*, Special Issue: Extracellular Biology. 34:199–230.

33. Klaenhammer, T.R., Barrangou, R., Buck, B. L., Azcarate-Peril M. A., Altermann E. (2005) Genomic features of lactic acid bacteria effecting bioprocessing and health *FEMS Microbiol. Rev.* 29(3), 393-409.

34. Kos, B., Šušković, J., Goreta, J., Matošić, S. (2000) Effect of Protectors on the Viability of *Lactobacillus acidophilus* M92 in Simulated Gastrointestinal Conditions*, Food Technol. Biotechnol.* 38 (2) 121-127.

35. Kos, B. (2001): Probiotic concept: *in vitro* investigation with selected lactic acid bacteria. PhD Thesis, Faculty of Food Technology and Biotechnology, University of Zagreb

36. Kos B, Šušković J, Vuković S, Šimpraga M, Frece J, Matošić S (2003) Adhesion and aggregation ability of probiotic strain *Lactobacillus acidophilus* M92. *J Appl Microbiol* 94, 981-987.

37. Kos, B., Šušković, J., Beganović, J., Gjuračić, K., Frece, J., Iannaccone, C., Canganella, F. (2008) Characterization of the three selected probiotic strains for the application in food industry. *World J. Microbiol. Biotechnol*. 24, 699-707.

38. Konstantinov, S.R., Smidt, H., de Vos WM, Bruijns SC, Singh SK, Valence F, Molle D, Lortal S, Altermann E, Klaenhammer TR, van Kooyk Y. (2008) S-layer protein A of *Lactobacillus acidophilus* NCFM regulates immature dendritic cell and T cell functions *Proc Natl Acad Sci U S A.* 105, 19474–19479.

39. Lebeer, S., Vanderleyden, J., De Keersmacker, S.C.J., (2008) Gene and Molecules of Lactobacilli Supporting Probiotic Action, *Microbiol Mol Bio Rev* 72(4), 728-764.

40. Leboš Pavunc A, Beganović J, Kos B, Uroić K, Blažić M, Šušković J (2012) Characterization and Application of Autochthonous Starter Cultures for Fresh Cheese Production. *Food Technol Biotechnol* 50(2), 141-151.

41. Lorca, G., Torino, M.I., Font de Valdez, G., Ljungh, A.A. (2002) Lactobacilli express cell surface proteins which mediate binding of immobilized collagen and fibronectin. *FEMS Microbiol. Lett.* 206, 31-37.

42. Lortal S, Van Heijenoort J, Gruber K, Sleytr UB. (1992) S-layer of *Lactobacillus helveticus* ATCC 12046: Isolation, chemical characterization and re-formation after extraction with lithium chloride. *J.General Microbiol.* 138, 611-618.

43. Ljungh, Å. and Wadström T. (2009) *Lactobacillus* Molecular Biology: From Genomics to Probiotics, Caister Academic Press, Norfolk, UK

44. Mobili, P., Gerbino, E. E. Tymczyszyn, E., Gómez-Zavaglia, A. (2010) S-layers in lactobacilli: structural characteristics and putative role in surface and probiotic properties of whole bacteria *In:* Current Research, Technology and Education Topics in Applied Microbiology and Microbial Biotechnology. Méndez-Vilas, A. (ed.). Badajoz, Spain: Formatex Research Center, pp. 1224-1234.

45. Maldonado, A., Jimenez-Diaz, R., Ruiz-Barba, J.L. (2004) Induction of plantaricin production in *Lactobacillus plantarum* NC8 after coculture with specific gram-positive bacteria is mediated by an autoinduction mechanism. *J. Bacteriol. 186*, 1556-1564.

46. McAuliffe, O, Cano, RJ, Klaenhammer, TR (2005) Genetic analysis of two bile salt hydrolase activities in *Lactobacillus acidophilus* NCFM. *Appl. Environ. Microbiol.* 71(8): 4925-4929.

47. Perdigón, G, Locascio, M, Medici, M, Pesce de Ruiz Holgado, A, Oliver, G (2003) Interaction of bifidobacteria with the gut and their influence in the immune function. *Biocell* 27:1–9.

48. Prado Acosta M., Palomino M.M., Allievi, M.C., Sanchez Rivas, C., Ruzal, S.M. (2008) Murein Hydrolase Activity in the Surface Layer of *Lactobacillus acidophilus* ATCC 4356 *Appl. Environ. Microbiol*. 74, 7824-7827.

49. Pretzer, G., Snel, J., Molenaar, D. & 7 other authors (2005) Biodiversity-based identification and functional characterization of the mannose-specific adhesin of *Lactobacillus plantarum*. *J Bacteriol* 187, 6128–6136.

50. Sánchez, B., Urdaci, M., A, Margolles, A. (2010) Extracellular proteins secreted by probiotic bacteria as mediators of effects that promote mucosa– bacteria interactions. *Microbiol.* 156, 3232–3242.

51. Sengupta, R., Altermann, E., Anderson, R. C., McNabb, W. C., Moughan, P. J., Roy, N. C. (2013) The Role of Cell Surface Architecture of Lactobacilli in Host-Microbe Interactions in the Gastrointestinal Tract Mediators of Inflammation http://dx.doi.org/10.1155/2013/237921

52. Servin, A.L., Coconnier, M.H. (2003) Adhesion of probiotic strains to the intestinal mucosa and interaction with pathogens. *Best Pract Res Clin Gastroenterol.* 17(5), 741-754.

53. Schneitz, C., Noutio, L., and Lounatmaa, K. (1993) Adhesion of *Lactobacillus acidophilus* to avian intestinal epithelial cells mediated by the crystalline bacterial cell surface layer (S-layer). *J. Appl. Bacteriol.* 74, 290-294.

54. Sillanpää, J., Martínez, B., Antikainen J., Toba, T., Kalkkinen, N., Tankka, S., Lounatmaa, K., Keränen, J., Höök, M., Westerlund Wikström, B., Pouwels, P.H., Korhonen, T. K. (2000), Characterization of the collagen binding S-layer protein CbsA of *Lactobacillus crispatus*. *J. Bacteriol.* 182, 6440-6450.

55. Smit, E., Oling, F., Demel, R., Martinez, B., Pouwels, P.H. (2001) The S-layer protein of *Lactobacillus acidophilus* ATCC 4356: identification and characterization of domains responsible for S-proteins assembly and cell wall binding. *J. Mol. Biol.* 305, 245-257.

56. Šušković, J. (1989): Effect of the nutrient medium composition on the kinetics of food-grade lactic acid formation; Master Thesis, Faculty of Food Technology and Biotechnology, University of Zagreb.

57. Šušković, J. (1996): The growth and probiotic effect of chosen lactic acid bacteria, PhD Thesis, Faculty of Food Technology and Biotechnology, University of Zagreb

58. Šušković, J., Kos, B., Goreta, J., Matošić, S. (2001) Role of lactic acid bacteria and bifidobacteria in synbiotic effect, *Food Technol. Biotechnol.* 39, 227-235.

59. Šušković, J., Kos, B., Frece, J., Beganović, J., Leboš Pavunc, A. (2009) Probiotički koncept – probiotici kao dodaci hrani i probiotici kao bioterapeutici. Hrvatski časopis za prehrambenu tehnologiju, biotehnologiju i nutricionizam. 4, 3-4; 77-84.

60. Šušković J., B. Kos, Beganović J., A. Leboš Pavunc, K. Habjanič, S. Matošić (2010) Antimicrobial Activity-The Most Important Property of Probiotic and Starter Lactic Acid Bacteria, *Food Technol. Biotechnol.* 48 (3) 296-307.

61. Tannnock, G. W. (2004) A special fondness for lactobacilli. *Appl. Environ. Microbiol*. 70, 3189-3194.

62. Uroić, K. (PhD thesis in progress) S-protein roles in probiotic lactic acid bacteria, Faculty of Food Technology and Biotechnology, University of Zagreb.

63. Ventura, M., Jankovic, I., Walker, D.C., Pridmore, R.D., and Zink, R. (2002) Identification abd characterization of novel surface proteins in *Lactobacillus johnsonni* and *Lactobacillius gaserri. Appl. Environ. Microbiol.* 68, 6172-6181.

64. Walter. J. (2005) The Microecology of Lactobacilli in the Gastrointestinal Tract. In. Probiotics and Prebiotics: Specific Aspects, G. W. Tannock, ed. (Norfolk, UK: Caister Academic Press).

65. Zoetendal, E.G., Cheng, B., Koike, S., Mackie, R.I. (2004) Molecular microbial ecology of the gastrointestinal tract: from phylogeny to function. *Curr. Issues Intest. Microbiol*. 5, 31-47.

Application of High Power Ultrasound in Inactivation of Microorganisms

Zoran Herceg*, Anet Režek Jambrak and Vesna Lelas *Minireview*

University of Zagreb, Faculty of Food Technology and Biotechnology,
Pierottijeva 6, 10000 Zagreb

High power ultrasound processing is a food processing method which has shown great potential in the food industry. Similar to heat treatment, high power ultrasound processing inactivates microorganisms and extends the shelf life of food products. Unlike heat treatments, high power ultrasound treatment can also maintain the quality of foods, with little effects on flavour and nutritional value. However, the use of ultrasound on its own in the food industry for bacterial destruction is currently unfeasible; however, the combination of ultrasound and heat and/or pressure shows considerable promise.

Key words:
High power ultrasound, Inactivation, Microorganism

Introduction

Technology and innovation are very important in the food manufacturing, first of all for the quality of the final product and also for safety and environmental issues. The most important trends in development of novel food processing technologies are relative to plant and to process. The design of energy saving equipment is an important issue in food processing, since electricity represents the main input of the process and its consumption reduction leads to better environmental and cost performances. The food industries still use heat through thermal processing operations (pasteurization, sterilization) in order to guarantee the microbiological safety of products. These traditional heating methods rely on the generation of heat outside the product to be heated, by combustion of fuels or by an electric resistive heater, and its transference into the product through conduction and convection mechanisms. These ways of processing are still limited due to considerable losses of heat on the surfaces of the equipment, reduction of heat transfer efficiency and thermal damage by overheating, due to the time required to conduct sufficient heat into the thermal centre of foods. Another important aspect that must be taken into account is the quality attributes (flavour and odour, visual apearence, color and texture,

*Corresponding author: zherceg@pbf.hr

nutrition value, absence of additives). Many food ingredients and products are well known to be thermally sensitive and can be damaged to chemical, physical and microbiological changes. Losses of some compounds, low production efficiency, time- and energy-consuming procedures such as prolonged heating and stirring may be encountered using these conventional food processing methods. These shortcomings have led to the continuous industrial interest in developing alternative – „green (or clean) and innovative“ techniques in processing and preservation of food which may be used to replace the severe heat-based methods that are commonly used. Recent advances in the search for such non-thermal processing methods led research to investigate the application of ultrasound. Ultrasound is an example of new technology and its application in food processing could lead to both these areas undergoing an improvement. (Chandrapala, J. et al., 2012)

The applications for high power ultrasound (HPU) in food processing are numerous and include degassing, extractions, induction of oxidation/reduction reactions, nucleation for crystallization processes, cleaning of organic/inorganic surfaces and porous interior structures, reducing the particle size and variability in liquid suspensions and the defouling of filters (Krešić et al., 2008). Also, ultrasound can pasteurize and preserve foods by inactivating many enzymes and microorganisms at mild temperature conditions, which can improve food quality in addition to guaranteeing stability and safety of foods.

Ultrasound mechanism

Ultrasonication is the application of high intensity sound waves at frequencies between 16 kHz and 100 MHz (Mason & Cordmas, 1996; Mason, 1998). The lowest frequency classification in the acoustic spectrum is infrasound that has a frequency range less than about 20 Hz. Audible sound is what human beings hear and has an approximate frequency range between 16 Hz and 18 kHz. The ultrasound frequency range starts at a frequency of about 20 kHz (Fig 1.). Ultrasound range can be divided into three different frequency ranges (Ashokkumar and Kentish, 2011):

- diagnostic ultrasound (1–10 MHz),
- high frequency ultrasound having a few hundred 100 kHz–1 MHz with low sound intensity (0.1–1 W cm^{-2}),
- low frequency power ultrasound in the kHz range (20–100 kHz) with high sound intensity (10–1000 W cm^{-2}) .

Diagnostic ultrasound involves low amplitude (higher frequency) propagation, which is concerned with the effect of the medium on the wave and is commonly

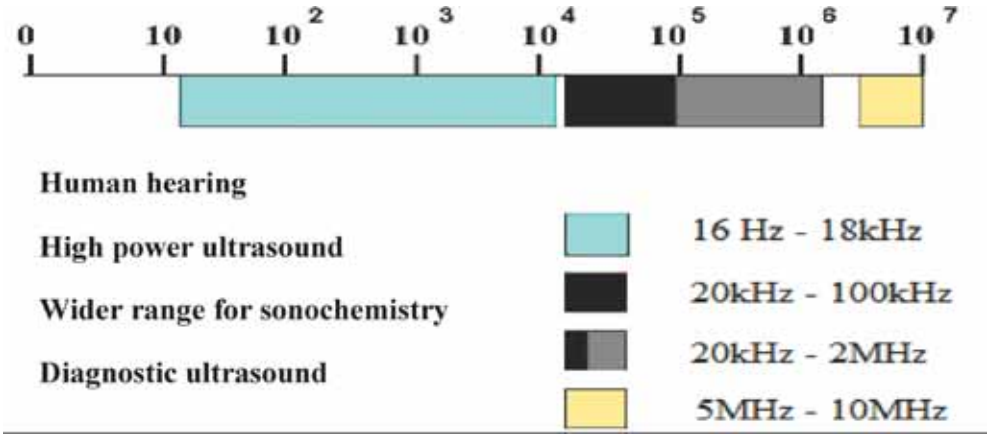


Fig. 1 – Range of ultrasound frequency (Leonelli, C., and Mason, T.J., 2010)

referred to as "low power" or "high frequency ultrasound". Typically, low amplitude waves are used to measure the velocity and absorption coefficient of the wave in a medium in the 1 to 10 MHz range. Useful industrial applications include texture, viscosity, and concentration measurements of many solid or liquid foods; composition determination of eggs, meats, fruits and vegetables, dairy, and other products.

High frequency ultrasound involves ultrasound frequency in the range of 100 kHz-1 MHz with low sound intensity (0.1-1 W cm^{-2}).

Power ultrasound involves high energy (low frequency) waves known as "power ultrasound". Frequency is between 20 and 100 kHz which is used for food processing as pre-treatment, in extractions, freezing, drying, defoaming, cleaning, depolymerisation, disaggregation, inactivation of microorganisms etc. Power ultrasound (20-100 kHz) can provide the mechanical effect of cavitation in liquid systems which can alter physical and chemical properties of food depending on the type of material involved. When ultrasound waves pass through a medium a series of compression and rarefaction waves on the molecules of the medium are produced. This will enforce a sinusoidal acoustic pressure (Pa) in addition to the hydrostatic pressure acting on the medium (Soria and Villamiel, 2010). If a large negative pressure (sufficiently below ambient) is applied to the liquid so that the distance between the molecules exceeds the critical molecular distance necessary to hold the liquid intact, the liquid will break down and the cavitation bubbles will be formed (O'Brien, 2007). These bubbles are formed from the gas nuclei within the fluid and are distributed throughout the liquid. After a period of few cycles, the bubbles will grow into a critical size which makes them unstable and violently collapse (Chemat et al., 2011).

Ultrasound and microbial inactivation

Traditionally heat treatment (pasteurization or sterilization) was a method of choice having the ability to destroy both micro-organisms and enzymes, the latter being responsible of food deterioration. However its effectiveness is dependent on the treatment temperature and time which leads to deterioration of functional properties, sensory characteristics (e.g. off flavor) and nutritional value of food products. Today's challenge is to combine simultaneously mild thermal preservation techniques with new applications for microbial destruction. The new technologies being developed insure the preservation of food without the use of preservatives, while keeping foods nutritional value and sensory characteristics unchanged and a high degree of safety. Less energy-intensive preservation method like high power ultrasound (HPU) are cost-efficient and environmentally friendly. It has been proved that high-intensity ultrasonic waves can rupture cells and denature enzymes, and that even low-intensity ultrasound is able to modify the metabolism of cells. In combination with heat, ultrasound can accelerate the rate of heat treatment of foods, thereby lessening the duration and intensity of thermal treatment and the resultant damage (Piyasena et al., 2003). Generally, most micro-organisms showed greater sensitivity to ultrasound at increased temperatures over 50°C, but, some authors claim that it is possible to inactivate microorganisms at temperatures of 40 ˚C (Herceg at al., 2013). Elevated temperature weakens the bacterial membrane, which enhances the effect of cavitation due to the ultrasound. In particular, the use of HPU has shown several advantages compared to heat pasteurization such as minimization of flavor loss in juices, greater homogeneity and significant energy savings (Herceg et al., 2012a, b).

Many researches have been done to understand the mechanism played by ultrasound on the disruption of microorganisms (Chandrapala et al., 2012; Earnshaw, Appleyard, & Hurst, 1995; Lopez-Malo, Guerrero & Alzamora, 1999; Raso et al., 1998; Cameronet al., 2008; Juraga et al., 2011) which has been explained by acoustic cavitation and its physical, mechanical and chemical effects that inactivate bacteria and deagglomerate bacterial clusters or flocs (Leighton, 2007). During the sonication process, when the cavitation bubbles, filled with gas or vapour, undergo irregular oscillations and finally implode. The imploding bubble create regions of very high temperature and pressure, reaching up to 5500 °C and 50,000 kPa, which can physically disrupt biological cells and denature enzymes. The effect of quick alternating pressures produced during cavitation disrupts microbial structures and causes the cell wall to break down. The high temperatures produced during cavitation may also have some effect, but as these temperature changes occur momentarily, only the liquid in the immediate surroundings is heated and therefore only a small number of cells are affected (Sala et al., 1995). Also, the extreme temperature conditions generated by a collapsing bubble can also lead to the formation of radical chemical species. Ultrasonic waves in water have been shown to form radicals by the following reaction due to homolytic cleavage:

$$H_2O \longrightarrow OH\cdot + H\cdot$$
$$OH\cdot + H\cdot \longrightarrow H_2O$$
$$2OH\cdot \longrightarrow H_2O_2$$
$$OH\cdot + OH\cdot \longrightarrow H_2 + O_2$$
$$2H\cdot \longrightarrow H_2$$
$$2OH\cdot \longleftrightarrow O\cdot + H_2O$$
$$2O\cdot \longleftrightarrow O_2$$
$$H_2O\cdot + 2H\cdot \longrightarrow H_2O + H_2$$
$$H_2O\cdot + H_2O\cdot + O_2 \longrightarrow 2H_2O_2$$
$$HO\cdot + H_2O \longrightarrow H_2O_2 + H\cdot$$

Additional reactions in the presence of oxygen.

$$O_2 \longrightarrow 2O\cdot$$
$$O_2 + O\cdot \longrightarrow O_3$$
$$O_2 + H\cdot \longrightarrow OOH\cdot (or\ OH\cdot + O)$$
$$O\cdot + OOH\cdot \longleftrightarrow OH\cdot + O_2$$
$$O\cdot + H_2O \longrightarrow 2OH\cdot$$

The hydroxy and hydroxyl radical formed in this reaction are highly reactive and rapidly interact with other radical or chemical species in solution. H· atoms are highly reducing in nature and OH· radicals are highly oxidizing. A common product of this reaction in water is hydrogen peroxide. However, scientists agree that the mechanism of microbial killing is mainly due to thinning of cell membranes, localized heating and production of free radicals (Chemat, 2011).

The effects, however, are not severe enough for a sufficient destruction of micro-organisms when using ultrasound (US – ultrasonications) alone at low temperature. To improve the microbial inactivation, ultrasound is combined with other treatments such as pressure (manosonic), heat (thermosonic), both pressure and heat (manothermosonic) and antimicrobials (Earnshaw, Appleyard, & Hurst, 1995; Pagan et al., 1999; López-Malo, Guerrero, & Alzamora, 1999; Piyasena et al., 2003; Raso & Barbosa-Canovas, 2003; Raso et al., 1998; Villamiel & de Jong, 2000).

Thermosonication (TS) - In this method, the product is subjected to ultrasound and moderate heat simultaneously. This technique shows the same inactivation level compared to the treatment without ultrasound at high temperature.

Manosonication (MS) -provides the possibility to inactivate enzymes and/or microorganisms by combining ultrasound with moderate pressure 100 - 300 kPa at low temperature.

Manothermosonication (MTS) - combines the ultrasound with moderate temperature and moderate pressure in order to inactivate enzymes and/or microorganisms. The ultrasound generates the cavitation or bubble implosion in the media. The simultaneous pressure treatment maximizes the intensity of the explosion, which increases the level of inactivation. Compared to HPU alone, these treatments are more energy-efficient and effective in killing microorganisms.

Pioneering work in this area was done by Ordonez et al. (1984) using ultrasound of 20 kHz and 160 W combined with temperatures ranging from 5 to 62 °C. The combination of heat and ultrasound was much more efficient with respect to treatment time and energy consumption compared to either treatment individually (Ordonez et al., 1984). McClements (1995) also suggested that inactivation of microbes using ultrasound is effective when used in combination with other decontamination techniques, such as heating, extremes of pH or chlorination. Raso and co-workers studied the influence of temperature and pressure on the lethality of ultrasound on the pathogenic bacteria *Yersinia enterocolitica*. Although ultrasound had a low lethal effect in ambient temperature and pressure, the lethality levels greatly increased with increasing static pressure and/or temperature. Raso et al. (1998) suggested an equation for predicting *D*-values when using manothermosonication. Assuming that heat and ultrasonic waves affect the medium independent of one another, the *D*-value of manothermosonication can be predicted using the following equation:

$$D_{\mathrm{MTS}} = (D_{\mathrm{T}} \times D_{\mathrm{MS}}) / (D_{\mathrm{T}} + D_{\mathrm{MS}})$$

where D_{MTS} is the decimal reduction time of manothermosonication (min), D_{T} is the decimal reduction of thermal treatment (min) and D_{MS} is the decimal reduction of manosonic treatment (min).

Herceg et al. (2013) suggested an equation for predicting *D*-values when using thermosonication assuming that ultrasound and temperature acted independently and that heat and ultrasound destruction of mikroorganisms were single reactions by first-order kinetics. In this way the logarithmic order of death of microorganisms would be expressed by the following equations: (developed model is based on the model Raso et al. (1998):

$$\frac{N_t^{TS}}{N_0} = \frac{N_t^{S}}{N_0} \cdot \frac{N_t^{T}}{N_0}$$

$$\log \frac{N_t^{TS}}{N_0} = \log \left(\frac{N_t^{S}}{N_0} \cdot \frac{N_t^{T}}{N_0} \right)$$

$$\log\frac{N_t^{TS}}{N_0} = \log\frac{N_t^{S}}{N_0} + \log\frac{N_t^{T}}{N_0}$$

$$-\frac{t}{D_{TS}} = -\frac{t}{D_S} - \frac{t}{D_T}$$

$$\frac{1}{D_{TS}} = \frac{1}{D_S} + \frac{1}{D_T}$$

$$\frac{1}{D_{TS}} = \frac{D_T + D_S}{D_S \cdot D_T}$$

$$D_{TS} = \frac{D_T \cdot D_S}{D_T + D_S}$$

where is: N_0 – number of microorganisms before treatment, N_t^T – number of microorganisms after time t and thermal processing, N_t^S number of microorganisms after time t and ultrasound treatmet, N_t^{TS} number of microorganisms after time t and thermal processing and ultrasound treatment, D_T decimal reduction time during thermal processing, D_S decimal reduction time during ultrasound treatment, D_{TS} decimal reduction time during thermal processing and ultrasound treatment.

There are often significant deviations in observed linearity of microbial inactivation by applying new methods of food processing. When survival curves are non-linear, the D value is usually determined by considering the linear portion of the survival curve. Over the years, a number of models have been proposed to describe these non-linear survival curves, such as the Cerf, modified Gompertz, log-logistic, Baranyi and Weibull models (Hassani et al., 2005; Ugarte-Romero et al., 2006, Lee et al, 2009). Among them, the Weibull model is gaining popularity due to its simplicity and flexibility. This model assumes that cells and spores in the population have different resistance and survival curve is just a form of cumulative distribution of lethal factors. Weibull model:

$$\log_{10}\frac{N}{N_0} = -bt^n$$

where b and n are the scale and shape factors.

Although the Weibull model describes non-linear survival curves better than the linear model, with one more parameter the Weibull model is intrinsically more complex. The concept of D and z values is no longer valid in these non-linear cases. It is proposed that the Weibull distribution parameters b and n are affected by external conditions, such as temperature, pH, and pressure, etc. (Peleg & Cole, 2000). Mattick et al. (2001) found that these two parameters were temperature de-

pendent and used rather complex empirical equations to describe the effect of temperature on the value of *b* and *n*..

It has been shown that microorganisms do not all react in the same way to ultrasound treatment.

Factors affecting the effectiveness of microbial inactivation are (Chemat et al., 2011):

- Amplitude of ultrasound waves.
- Exposure or contact time.
- Volume of food processed.
- Composition of food.
- Treatment temperature.

Also, the effectiveness of ultrasound on the inactivation of microorganisms is affected by type, shape and size of the microorganisms. Herceg et al. (2012a) was investigated of the effect combination of ultrasound and heat treatment *vs* ultrasound treatment alone on the inactivation of *Escherichia coli* and *Staphylococcus aureus* in milk. The parameters that seem to substantially affect the inactivation of *E. coli* and *S. aureus* in milk are the amplitude of the ultrasonic waves, the exposure/contact time with the microorganisms, and the temperature of the treatment. It was found that Gram-negative bacteria (*E. coli*) are more susceptible to the ultrasonic treatment than the Gram-positive ones (*S. aureus*). Gram-positive bacteria are known to be more resistant than gram-negative ones, possibly because of their thicker cell wall which provides them a better protection against ultrasound effects. According to the literature differences in cell sensitivity could also be due to the more tightly adherent layer of peptidoglucans in gram-positive cells. However, it is probable that a significant impact on the size of the cells inactivate microorganisms (Table 1.). Larger cells are more sensitive than the small ones. This is probably due to their larger surface area. Concerning the shape of the micro-organisms, cocci are more resistant than bacilli due to the relationship of cell surface and volume. Also, resistance of different species to ultrasound differs widely. Sporulated microorganisms are much more resistant than vegetative ones and fungi are more resistant in general than vegetative bacteria. The most commonly applied frequency ultrasound is at 20 kHz for microbial inactivation. The resistance to ultrasound treatment at this frequency of spores, and Gram-positive and coccal cells are higher than vegetative, Gram-negative and rod-shaped bacteria (Feng, Yang, & Hielscher, 2008). In addition, it also varies among different strains. For example, *Escherichia coli* and *Saccharomyces cerevisiae* were reduced bymore than 99% after ultrasonication, whereas *Lactobacillus acidophilus* was reduced by 72% and 84% depending on the media used (Cameron, McMaster, & Britz, 2008).

Table 1 – Structure of cell wall and size of cell like factors affecting the sensitivity of the bacteria (Chemat, F. et al., 2011).

Type of microorganism	Structure of cell wall	Cell size (µm)	Shape
Escherichia coli	Gram – negative	1,5 x 6	Straigts rods
Staphylococcus aureus	Gram – positive	0.8 x 1	Irregular coccoid
Salmonella typhimurium	Gram – negative	1.5 x 5	Straigts rods
Listeria monocytogenes	Gram – positive	0.5 x 2	Shorts rods
Bacillus subtilis	Gram – positive	0.5 – 2.5 x 1.5 – 10	Rods with rounded or squared ends

Since molds and yeasts are in general more resistant to high intensity ultrasound and not enough information about mold spores is available. In terms of microbial resistance more research is necessary about the potential enhancement of the ultrasound in combination with other preservation factors, among them antimicrobials, especially for fungi Lopez-Malo et al. (2005) demonstrated that thermosonication could effectively inactivate *Penicillium digitatum.* Thermal and thermosonication treatments were evaluated to determine optimal pH, water activity (a_w), temperature and ultrasonic amplitudes. At an a_w of 0.99, increasing ultrasonic amplitude and decreasing pH resulted in a decrease in *D*-values. When pH was kept constant, a higher a_w resulted in a lower *D*-value. It was found that when thermosonication was used, lower *D*-values were obtained when compared to heat treatment without ultrasound. The authors did not provide any numerical data for the *D*-values or the composition of the laboratory broth they used. Inactivation of *P. digitatum* as well as *Aspergillus niger* spores using ultrasonic treatment was studied by Jimenez-Munguia et al. (2001). Boiling chips and air bubbles were added to the treatment medium (Sabourand broth) to determine their effect on the inactivation. In general, *A. niger* (sonicated at 45 °C) showed lower *D*-values than *P. digitatum* (sonicated at 40 °C). Boiling chips and bubbles enhanced the effect of the cavitation from the sonication, reducing *D*-values further. This enhanced cavitation effect could be applied to sonication treatment processes to increase efficiency.

Conclusions

With the growing knowledge of the importance of food in maintaining the human health, the food industry is faced with increasing challenges to develop new non-thermal food treatment technologies that would prevent and mitigate food contamination while retaining nutritional and functional properties of the treated food. Ultrasound is one of the more advanced food technologies, it can be applied not

only to improve the quality and safety of processed foods but offers the potential for developing new products with unique functionality. However, the use of ultrasound on its own in the food industry for bacterial destruction is currently unfeasible; however, the combination of ultrasound and heat and/or pressure shows considerable promise. Ultrasonic processing is still in its infancy and requires a great deal of future research in order to develop the technology on an industrial scale, and to more fully elucidate the effect of ultrasound on the properties of foods.

References

1. Ashokkumar, M., and Kentish, S., (2011). The physical and chemical effects of ultrasound In Ultrasoiund technologies for food and bioprocessing. Feng, H., Barobosa-cànovas, G. V., Weiss, J. (ed). Springer, NY, USA. pp1-105.
2. Cameron M., McMaster L.D., Britz T.J., (2008) Electron microscopic analysis of dairy microbes inactivated by ultrasound, *Ultrason. Sonochem. 15* 960–964.
3. Chandrapala, J., Oliver, C., Kentish, S., Ashokkumar, M. (2012) Ultrasonics in food processing. *Ultrasonics Sonochemistry* 19, 975–983.
4. Chemat, F., Huma, Z., Kamran Khan, M. (2011). Applications of ultrasound in food technology: Processing, preservation and extraction. *Ultrasonics Sonochemistry*, *18(4)*, *813-83.*
5. Earnshaw R.G., Appleyard J. & Hurst R.M. (1995). Understanding physical inactivation process: combined preservation opportunities using heat, ultrasound and pressure. International Journal of Food Microbiology, 28, 197-219.
6. Feng H., Yang W., Hielscher T. (2008) Power ultrasound. Food Science and Technology International, 14 (5), 433.
7. Hassani M, Álvarez I, Raso J, Condón S, Pagán R. (2005). Comparing predicting models for heat inactivation of *Listeria monocytogenes* and *Pseudomonas aeruginosa* at different pH. *Int J Food Microbiol* 100:213–22.
8. Herceg Z., Režek Jambrak A., Lelas V., Mededovic Thagard S. (2012a) The Effect of High Intensity Ultrasound Treatment on the Amount of Staphylococcus aureus and Escherichia coli in Milk, *Food Technol. Biotechnol. 50* (1) 46–52
9. Herceg Z., Juraga E., Sobota Šalamon B., (2012b) Inactivation of mesophilic bacteria in milk by means of high intensity ultrasound using response surface methodology, *Czech J. Food Sci*. 30 108-117.
10. Herceg Z., Markov K., Sobota Šalamon B., Režek Jambrak A., Vukušić T., (2013) Effect of high intensity ultrasound treatment on growth of food spoilage bacteria, *Food Technol. Biotechnol. 51* (3) 352–359
11. Jiménez-Munguía M.T., Arce-García M.R., Argaiz A., Palou E. & López-Malo A. 2001. Mold spore inactivation during cavitation due to ultrasound treatments. In Institute of Food Technologists Annual Meeting, New Orleans, USA.
12. Juraga E., Sobota Šalamon B., Herceg Z., Režek Jambrak A. (2011) Application of high intensity ultrasound treatment on Enterobacteriae count in milk, Mljekarstvo 61 (2), 131-140.
13. Krešić, G., Lelas, V.,Režek Jambrak, A., Herceg, Z., Rimac Brnčić, S. (2008) Influences of novel food processing technologies on the rheological and thermophysical properties of whey proteins. *Journal of Food Engineering*. 87 (1), 64–73.

14. Lee H., Zhou B., Liang W., Feng H., Martin S. E. Inactivation of Escherichia coli cells with sonication, manosonication, thermosonication, and manothermosonication: Microbial responses and kinetics modeling (2009) *Journal of Food Engineering* 93 354–364.

15. Leighton, T.G. (2007). What is ultrasound? Progress in Biophysics and Molecular Biology 93, 3–83.

16. Leonelli, C., and Mason, T.J. (2010) Microwave and Ultrasonic processing: now a realistic option for industry. *Chemical Engineering and Processing*, 885-900

17. López-Malo A., Guerrero S., Alzamora S. M., (1999) *Saccharomyces cerevisiae* thermal inactivation kinetics combined with ultrasound, *J. Food Prot. 62* 1215-1217.

18. *López-Malo,* A., *Palou,* E., *Parish,* M. E., *and Davidson,* P. M., (2005), Methods for activity assay and evaluation of results, in: Antimicrobials in Food, 3rd edition, CRC Press, New York, USA, 429-451.

19. Mason, T. J. (1998). Power ultrasound in food processing – the way forward. In: Ultrasound in food processing. M. J. W. Povey & T. J. Mason (Eds.), London, UK: Thomson Science, 105–126.

20. Mason, T. J., & Cordmas, E. (1996). Ultrasonic intensification of chemical processing and related operations – a review. Transactions of the Institute of Chemical Engineers, 74 (A), 511–516.

21. Mattick, K. L., Legan, J. D., Humphrey, T. J., & Peleg, M. (2001). Calculating Salmonella inactivation in non-isothermal heat treatments from isothermal non-linear survival curves. *Journal of Food Protection*, 64, 606–613.

22. McClements, D. J. (1995) Advances in applications of ultrasound in food analysis and processing. *Trends in Food Science and Technology* **6**, 293-299.

23. O'Brien, W.D. Jr. (2007) Ultrasound–biophysics mechanisms. *Progress in Biophysics and Molecular Biology* 93 (2007) 212–255.

24. Ordonez, J. A., Sanz, B., Hernandez, P. E., Lopez-Lorenzo (1984) A note on the effect of combined ultrasonic and heath treatments on the survival of thermoduric streptococci. *Journal of Applied Bacteriology* **54**, 175-177.

25. R. Págan, P. Maňas, J. Raso, S. Condon, (1999). Bacterial resistance to ultrasonic waves under pressure at nonlethal (manosonication) and lethal (manothermosonication) temperatures, *Appl. Envionr. Microbiol. 65,* 297-300.

26. Patist, A. and Bates, D. (2008). Ultrasonic innovations in the food industry: From the laboratory to commercial production *Innovative Food Science and Emerging Technologies* 9, 147–154.

27. Povey, M., and Mason, T. (1998). *Ultrasound in food processing*. London, Blackie Academic and Professional.

28. Peleg, M., & Cole, M. B. (2000). Estimating the survival of Clostridium botulinum spores during heat treatments. Journal of Food Protection, 63, 190– 195.

29. Piyasena P., Mohareb E., McKellar R.C., (2003) Inactivation of microbes using ultrasound: a review, *Int. J. Food Microbiol. 87* 207–216.

30. Raso, J., & Barbosa-Canovas, G. V. (2003). Nonthermal preservation of foods using combined processing techniques. *Critical Reviews of Food Science &. Nutrition*, 43(3), 265-285.

31. Raso, J., Pagan, R., Condon, S., & Sala, F. J. (1998). Influence of temperature on the lethality of ultrasound. *Applied Environmental Microbiology*, 64, 465-471.

32. Sala F.J., Burgos J., Condón S., Lopez P., Raso J. (1995) Effect of Heat and Ultrasound on Microorganisms and Enzymes. In:G.W. Gould (Ed.), *New Methods of Food Preservation.* Aspen Publishers, Gaithersburg, MD, USA, 176-204

33. Soria, A. C., & Villamiel, M. (2010). Effect of ultrasound on the technological properties and bioactivity of foods: a review. *Trends in Food Science and Technology*, 21, 323-331.

34. Ugarte-Romero E., Feng H., Scott E. M., Cadwallader K. R., Robinson S. J. (2006) Inactivation of *Escherichia coli* with Power Ultrasound in Apple Cider, *Food Engineering and Physical Properties* 2 (71) 102-108.

35. Villamiel, M., & de Jong, P. (2000). Inactivation of *Pseudomonas fluorescens* and *Streptococcus thermophilus* in Trypticase soy broth and total bacteria in milk by continuous-flow ultrasonic treatment and conventional heating *Journal of Food Engineering 45* 171-179.

Endocrine Disruptors and Animal-Free Toxicology

Teuta Murati, Branimir Šimić, Jasna Kniewald and Ivana Kmetič* *Review*

University of Zagreb, Faculty of Food Technology and Biotechnology,
Laboratory for Toxicology, 10000 Zagreb, Pierotti St. 6, Croatia

Endocrine disruptors, hormonally active compounds which can modulate the endocrine and immune systems, have captured public's attention like no other toxicity concern since the publication of Rachel Carlson's *Silent Spring* in 1962. Because the endocrine system is vital to reproductive health and the maintenance of species, the research of thousands of chemicals produced and used in commerce that may damage this system, remains very high on the scientific agenda. For decades, toxicological risk assessment has used animal experiments as the main approach to ensure human health and safety of the environment, respecting the so-called 3Rs (Reduce, Replace, Refine) principle. Beside ethical and economic considerations, there are also scientific reasons why the future studies are seen in the strength of development and incorporation of stepwise testing strategies, combining experimental data from a range of alternative methods (physicochemical techniques, computerized modelling based on quantitative structure-activity relationships – QSAR, the omics technologies, metabolic and kinetic modelling and *in vitro* approach). The use of *in vitro* systems (subcellular systems, primary cell cultures, cell lines, stem cells, whole tissues and perfused organs) provide a detailed insight into the mechanisms of toxicity. At the same time, high-throughput and high content measurements on various cell models provide a sensitive and robust approach for screening new chemical entities. To fill remaining gaps of knowledge, targeted testing in animals would then be performed as an additional step. This would change toxicology from being a predominantly observational craft and regulatory support discipline back to a natural science with all its dimensions. According to the fact that Croatia is from July 1 a member of EU, all appropriate testing regulations must be now harmonized. Concerning the toxicity validation it means that animal-free testing methods will be obligated and applied in Croatia without any further delay as much as it is possible.

Key words:
endocrine disrupting chemicals, the 3Rs, animal-free toxicology

*Corresponding author: ikmetic@pbf.hr

Intoduction

Our environment has radically changed over the past century, especially in developed countries. For a half-century of intensification of industrial era, more than 5 million man-made chemicals have been released in the environment without recycling[1]. These products were often designed either to be stable, as being rather insoluble (plasticizers, PCBs, diverse oil or other industrial residues like heavy metals...), and/or to be penetrating and active on the physiology of the organisms (drugs, pesticides, etc.). Because of their properties, these agents might become excellent candidates for disruption of hormone homeostatic balance in animals and humans and become so-called endocrine disrupting chemicals (EDCs), *xenobiotic, environmental hormones, hormonally active agents* or *environmental signals*[2]. Chemicals classified as EDCs, some of them banned and some still in use, produce their effects by mimicking, antagonizing or altering endogenous steroid levels by changing rates of their synthesis or metabolism and/or expression or action at receptor targets[3]. Because of growing concerns among the public at large on the potential adverse effects that may result from exposure to EDCs (potential to affect immune function and reproduction, changes in neurosteroid levels, behaviour and memory), endocrine disruption remains very high on the scientific agenda and one of the most controversial contemporary environmental issues[4]. Current toxicological risk assessment to ensure human health and the safety of the environment is still based predominantly on animal studies. Because animal-based testing is expensive and time-consuming, morally and ethically troubling, and most significantly, often a poor predictor of human toxicity, recently there is a welcome trend of combining *in vitro* and *in silico* models to predict chemical safety. One of the most comprehensive strategies to make toxicology largely independent of animal studies is "*Toxicity testing in the 21st century: a vision and a strategy*," as put forward by the National Research Council[5]. A new concept of combining a largely heterogeneous group of assays, providing information at different levels of complexity, with different throughput rates, and possibly with different information value could fill knowledge gaps and improve the overall risk assessment of chemicals for which little is known before they reach the market and would contribute to an animal-free risk assessment.

What are endocrine disrupting chemicals?

As the name suggests, EDCs are mainly anthropogenic substances that can interfere with the endocrine systems of living organisms. An EDC was defined by the U.S. Environmental Protection Agency (EPA) as "an exogenous agent that interferes with synthesis, secretion, transport, metabolism, binding action,

or elimination of natural blood-borne hormones that are present in the body and are responsible for homeostasis, reproduction and developmental process"[6]. The group of molecules identified as endocrine disruptors is highly heterogeneous and is not a defined group of substances. Potential EDCs include some man-made chemicals and by-products like pesticides (e.g. dichlorodiphenyltrichloroethane – DDT, methoxychlor, atrazine, chlorpyrifos, endosulfan), industrial chemicals (e.g. polychlorinated biphenyls – PCBs, polybrominated biphenyls – PBBs, dioxins), plastics (e.g. bisphenol A – BPA), plasticizers (e.g. phthalates), parabens, pharmaceuticals (e.g. drug estrogens – birth control pills, diethylstilbestrol – DES, cimetidine), metals (e.g. cadmium, lead, mercury), breakdown products of detergents and associated surfactants, including nonylphenol and octylphenol. Natural chemicals produced by plants (the so-called phytoestrogens, such as genistein or coumestrol) and certain fungi can also act as endocrine disruptors[6,7,8].

These are substances that can be structurally, but not necessarily, resemble to natural hormones. They not only affect endocrine and reproductive functions but also central nervous system and thyroid, the immune system, the cardiovascular system, the digestive system, can cause prostate and breast cancer and obesity, congenital anomalies and can act as epigenetic modulators[1,6,9,10]. Dose, time, and even exposure period to EDCs are important factors to take into consideration. Even very low levels of exposure, particularly during a critical developmental period may cause damage or abnormalities. Surprisingly, low doses may even exert more potent effects than higher doses. Furthermore, effects of different classes of EDCs may be additive or even synergistic[6]. Moreover, effect of developmental exposure may not be immediately apparent early in life but manifested in adulthood or during aging.

Exposure to potential EDCs of humans and wildlife

EDCs enter the environment during their production, use and disposal. Some of them may be released into the environment intentionally (e.g. pesticides), but for most environmental contaminants release is unintentional (e.g. „dioxin-like" chemicals like polychlorinated dibenzo-*para*-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) are formed unintentionally as by-products in a variety of industrial and combustion processes)[11]. Today, the use of PCBs is prohibited according to Stockholm Convention and the current sources are primarily landfills of transformers, capacitors and inadequate disposal of waste in open areas. Although their concentration in the environment gradually decreases, in Croatia risk is still present due to military destructions during the War of independence (1991-1995), when many electric powers, in-

dustrial and other facilities were damaged or destroyed resulting with the leakage of PCBs[12].

Larger emissions of EDCs occur when chemicals are incorporated into materials and products – such as plastics, furniture, carpets and electrical equipment and when these goods are used, than when raw chemicals are manufactured[13].

Many EDCs are found in a myriad of household products. BPA is present in polycarbonate plastics, including beverage and food storage containers, and epoxy resins that line the interior of metal cans[3]. Textiles can contain contaminants, such as flame retardants, including tetrabromobisphenol A (TBBPA) and polybrominated diphenyl ethers (PBDE).

Some individuals have been exposed to contaminants with adverse effects due to medical (diethylstilbestrol, DES) or dental (diglycidyl methacrylate) interventions. Synthetic estrogens from contraceptive pills, such as ethynilestradiol (EE2), are commonly found in surface water, because of their widespread use[3]. Other environmental sources of EDCs include leakage from agricultural fields and distribution via sewage sludge, emissions from urban areas and long-range transport via air and ocean[11]. These substances are found even in places where they are neither in use or produced, like deserted, cold continent Antarctica[1, 14]. Recently, nitrates have become one of the potential endocrine disruptors because they may disrupt gonadal steroidogenesis and thyroid function[10].

Because of their chemical properties, primarily stability and lipophilicity, most of potential EDCs are persistent in the environment and their bioaccumulation increases through food chains. Some EDCs are less persistent in the environment and do not remain in humans and wildlife for very long but they are a concern because exposure to them can be continuous[13].

Exposure of potential EDCs in humans and wildlife can occur via air, water, soil, sediment, food and consumer products. The chemical may then enter the organism by ingestion, inhalation or skin contact[11]. The major exposure pathways for humans to many EDCs are via food and drinking water. Food rich in animal grease like meat, fish or eggs, often contains increased amounts of such contaminants[1]. The isoflavonoid phytoestrogens are found in soy and legumes, the lignanes in grains and many fruits and vegetables, and the coumestans in clover and alfalfa[11].

Most EDCs can be transferred to the fetus and the newborn through the placenta or breast milk, respectively. Some chemicals affect the endocrine system in their original form, whereas others undergo metabolic transformations in the body or are abiotically transformed to forms that make them hormonally active[13].

Mechanisms of action

The exact mode of action(s) of endocrine modulators has not been fully elucidated. In the literature there are at least five mechanisms that explain how endocrine modulators interfere with the normal role of natural hormones and function of the endocrine system:

1) compounds that have very similar structures to hormones are able to bind to cellular receptors which consequently may have unexpected activity in the cell;
2) some EDCs can block the binding sites of the receptor so that natural hormones cannot bind to them, with the consequence of disrupting normal cellular activity and hormonal status;
3) some EDCs can stimulate the creation of extra receptor sites in the cell, which consequently leads to a stronger effect of hormones and increased cellular immune responses;
4) some EDCs can directly or indirectly interact with natural hormones, changing the hormones message and thus altering cell activity;
5) some EDCs can alter the natural pattern of hormone synthesis and metabolism, thus affecting circulating hormone levels and disrupting hormonal balance of the organism[15].

Most EDCs may interact with the effects of lipid (steroid) or amino acid derived (thyroid) hormones, while a few interact with peptide/protein hormone synthesis or signalling molecules[16, 17]. Interferences of EDCs with steroid biosynthesis may result in impaired reproduction, alterations in (sexual) differentiation, growth and development as well as the development of certain cancers. Steroid hormone synthesis is controlled by the activity of several highly substrate-selective cytochrome P450 enzymes and a number of steroid dehydrogenases and reductases[18, 19]. The androgen – estrogen balance is crucial for oocyte maturation and spermatogenesis[1]. The key enzyme which controls androgen/estrogen ratio is aromatase, an isoform (CYP19) of the cytochrome P450 family of enzymes[20]. This enzyme converts androgens to estrogens and represents a possible new target for xenobiotics and has been the subject of many studies into the mechanisms by which chemicals interfere with sex steroid hormone homeostasis and function, often related to (de)feminization and (de)masculinization processes[1, 19]. Such effects of EDCs were originally observed in non-mammalian species. Feminisation occurs in fish (manifested by the production of the egg protein vitellogenin) when they are exposed to endocrine disruptor-containing sewage effluent and in the amphibian *Xenopus laevis* exposed to octyl- and nonyl-phenols and BPA at submicromolar concentrations[20]. Studies with triazine herbicide atrazine have shown demasculinization of male gonads producing testicular lesions associated with reduced germ cell numbers in teleost fish, amphibians, reptiles and mammals, and induced partial and/or complete feminiza-

tion in fish, amphibians and reptiles[21]. Masculinisation can also occur. The compound tributyltin, once used to prevent fouling of ships' timbers, is an aromatase inhibitor which causes significant masculinisation of female zebra fish and sperm damage (lower levels of motility or loss of flagella) at very low concentrations[20].

In rats and mice exposed to EDCs hypospadia, cryptorchidism, microphallus and prostate cancer have been observed in males, and abnormalities in the mammary glands and reproductive tracts (including increased risk of cancers) in females[20]. Some pesticides in current use appear to have also effects on the endocrine system. Various pyrethroids have been demonstrated to have weak antiandrogenic, antiestrogenic, or estrogenic activity, and chlorpyrifos has been shown to affect thyroid hormones in animal and human studies[22]. Health effect studies for many organochlorines such as DDT, chlordane and methoxychlor suggest neurotoxicity, effects on developing reproductive systems, effects on lactation, and development of various cancers, including breast cancer. An organochlorine insecticide lindane may cause the impairment of female fertility by altering ovarian development and function, and implantation by altering endometrial function through its estrogenic activity[23]. Numerous studies on animals confirmed that PCBs cause behaviour disturbances and reduced learning ability, but also indicate that human exposure to PCBs in the earliest years of life also causes neurobehavioral disruption[3, 12].

Exposure to xenobiotic can induce a state of immunodeficiency, even immunosuppression, inflammation, hypersensitivity or allergy[1].

Children are not „small adults"

Exposure to chemicals, especially those with hormone like activity, is more dangerous if it occurs during specific "critical periods" of life, such as intrauterine, perinatal, and juvenile or puberty periods, than in other periods when they may cause irreversible long-lasting consequences[3, 24, 25]. The fetal period is extremely vulnerable and most sensitive stage of life. The effects of exposure to chemicals during that stage may not appear immediately after exposure but long afterward, even in puberty or adulthood, and is known as the delayed long-term effect[26]. To unique sensitivity of these periods contributes rapid cell proliferation and cell differentiation as well as complex patterns of cell signalling and cell migration.

Infants are mainly exposed to EDCs via breast milk and may consume much higher levels of contaminants per kilogram of body weight during the lactation period than during later stages of life[11]. Further, fetuses and neonates have high metabolic

rates, undeveloped liver detoxifying mechanisms, and undifferentiated immune systems as compared to adults making them more prone to chemical insult.

Lessons learned from history

In an attempt to prevent miscarriages caused by progesterone deficiency and other complications of pregnancy, between 1940 and 1971, DES, a synthetic nonsteroidal compound with estrogenic activity, was prescribed to several million pregnant women [25, 27]. DES became one of the first examples of a transplacental toxicant in humans but the abnormalities caused by DES were not detectable until later in life. DES was linked to more frequent benign reproductive tract problems in the DES-exposed daughters such as reproductive organ malformation and dysfunction, poor pregnancy outcome, and immune system disorders. Similarly, in the DES-exposed male offspring hypospadias, microphallus, retained testes and increased genital-urinary inflammation were reported to result from prenatal DES exposure[25]. It was used until 1971 when it was showed that DES caused an increased incidence of vaginal clear cell adenocarcinoma (CCA) in the daughters of women treated with the drug and US Food and Drug Administration (FDA) advised physicians to stop prescribing DES. In 1978 the FDA withdrew approval for the use of DES but it continued to be used in clinical trials for treatment of prostate and breast cancer until the late 1990s[27]. The DES episode is a salient reminder of the potential toxicity and carcinogenicity that may be caused by developmental exposure to hormonally active chemicals.

The introduction of synthetic organochlorine pesticide DDT revolutionised agricultural production and has been credited with the eradication of malaria from the United States and Europe. But it was the first intentionally released chemical found to be estrogenic. In 1949, aviation crop dusters handling DDT were found to have reduced sperm counts[28]. Findings that DDT and its metabolites have human estrogenic activity and dichlorodiphenyldichloroethylene – DDE, the key DDT metabolite, to act as an androgen antagonist, as well as a trend for decreasing semen quality in the general human community following the introduction of DDT, have prompted suggestions that environmental exposure to organochlorines may be causing endocrine disruption in human populations[29]. Although DDT use has been restricted or banned in most developed countries since the early 1970s, exposure to this pesticide remains widespread. Its chemical stability and an associated lipophilicity result in DDT, and also DDE, being persistent in both the environment and the human body and slowly eliminated by most living beings[29].

It is quite thought-provoking that although the discovery that DDT had insecticide properties earned Müller the Nobel Prize and the discoverer of DES, E. C. Doods, received English knighthood, both these substances have caused a lot of damage to human health[26].

From Rachel to REACH

Since the publication of Rachel Carlson, an American writer, scientist and ecologist, from 1962 called *Silent Spring*, in which she intent to warn the public of the dangers associated with pesticide use, especially DDT, attention to this area of investigation has grown. Because the endocrine system is vital to reproductive health and the maintenance of species, the research of thousands of chemicals that may damage this system, remains very high on the scientific agenda. Since then, attention to this area of investigation has grown, which confirms the enormous rise in the number of scientific papers related to this topic.

At present, the major driver for a rethinking of toxicity testing in Europe is the REACH legislation[30]. The **R**egistration, **E**valuation, **A**uthorisation and Restriction of **Ch**emical substances (REACH) is a regulation of the European Union, adopted to improve the protection of human health and the environment through the better and earlier identification of the intrinsic properties of chemical substances and their toxicological and ecotoxicological properties[31]. The law entered into force on the 1st of June 2007. While in the past a set of mainly animal tests had to be provided, now (for both existing and especially new industrial chemicals) new and existing approaches are combined in order to optimise information generation, making use also of *in vitro*, *in silico* and read-across data from similar compounds in a preliminary hazard evaluation[30].

The 3Rs

For decades, the mantra in the world of laboratory animals has been the so-called 3Rs, which encourages researchers to *Reduce* the number of animals they use, *Refine* the assays to reduce distress, pain and suffering, and ultimately, *Replace* animals with alternative methods. The concept of 3Rs was first described in 1959, in *The Principles of Humane Experimental Technique*, classic "ahead of its time" publication by British researchers W. M. S. Russell, a zoologist, and R. L. Burch, a microbiologist[32, 33]. Until the 1970s there was no interest to start the developing of 3Rs. Since then, the concept has become a very important one in scientific research

and testing involving the use of animals. *Reduction* of animal numbers can be achieved by improved experimental design and statistical analysis, and thorough literature reviews so that unnecessary duplication of animal studies is avoided[32, 34]. The way experiments are carried out must be refined to make sure animals suffer as little as possible. This includes better living conditions and improvements to procedures which minimise pain and suffering and improves animal welfare[32, 33]. *Replacement* alternatives include various methods (physicochemical techniques; computerized modelling based on quantitative structure activity relationships – QSAR; the omics technologies – technologies that measure some characteristic of a large family of cellular molecules, such as genes, proteins, or small metabolites, e.g. genomics, proteomics, metabolomics, transcriptomics, glycomics, lipomics; metabolic and kinetic modelling, micro array technology and *in vitro* approach)[35, 36, 37, 38]. Emerging QSAR (mathematical descriptions of the relationships between the physicochemical properties of molecules and their biological activities) and other *in silico* models will become increasingly useful for identifying likely metabolites and predicting potential target tissues for toxicity, so that the appropriate assays of *in vitro* effects can be selected[36, 39]. From the modest origins and use of animal cells at a laboratory scale for more than 100 years, animal cell culture technology has evolved into a modern technology based on scientific and engineering principles. The use of animal cell culture has lead to important achievements in the development and production of vaccines and recombinant proteins, *in vitro* tissues and organs. In addition, applications of animal cell culture now underpin specific scientific fields such as cell and gene therapy, *in vitro* toxicology and physiology, production of biopesticides, nanobiotechnology. The use of three-dimensional (3D) *in vitro* cell cultures which more closely resemble the architectural microenvironment of natural tissue has been increasingly used as a potential link to bridge the gap between monolayer cultures and animal model studies. As knowledge about properties of stem cells expands, their use as unique tool for the treatment of wide range of diseases notably increases. The use of *in vitro* systems (subcellular systems, primary cell cultures, cell lines, stem cells, whole tissues and perfused organs) can provide information on the nature and concentration response of the toxic effects of the chemicals and a detailed insight into the mechanisms of toxicity[35].

The Laboratory for Toxicology was established at the Faculty of Food Technology and Biotechnology University of Zagreb in 1989. From the very beginning the activities were oriented on the introduction of animal tissue cultures and later from 1998 of cell culture technology in studying of environmental toxicant effects on reproductive processes in vertebrates and avian. Published papers were mainly oriented on influence of atrazine on reproductive processes and were among the first in that topic which put attention on the presence of dangerous amounts of atrazine in the environment[16]. At the same time, high-throughput and high content measurements on various cell models provide a sensitive and robust approach for screening new chemical entities. Gene reporter assays using luciferase or beta-lactamase have

been developed and can easily be used for screening potential endocrine disruptors in humans and wildlife. One mechanism of action of EDCs is to act directly as ligands to steroid hormone nuclear receptors, in particular estrogen, androgen and thyroid hormone receptors[40]. This developed test can easily be used to screen for both agonist and antagonist activity for these as well as other nuclear receptors.

Scientific validation and regulatory acceptance

Validation is the process by which the reliability and relevance of a procedure are established and evaluated for a specific purpose[35, 41]. The relevance of a procedure refers to the scientific value and the practical usefulness of the results it provides, whereas reliability is concerned with the reproducibility of these results within and between laboratories and over time, in relation to a clearly defined and specific purpose[42]. A process of validation is not a process to develop new approaches, optimize approaches, or compare one approach to another. It is a process that verifies the method or procedure in question performs as intended to give account of test characteristics such as precision, limit of detection, accuracy, specificity, sensitivity, robustness and transferability[41, 43]. The challenges of new technologies (omics, high-throughput screening, high content methods) urge to adapt and expand the concepts of validation.

The European Commission's involvement in activities targeted to the validation of alternative approaches to animal testing started in 1991, with the launch of ECVAM (the European Centre for the Validation of Alternative Methods). As from 2011, ECVAM's tasks are assigned to EURL ECVAM (European Union Reference Laboratory for alternatives to animal testing). The aim of EURL ECVAM, beside to co-ordinate the independent evaluation of the relevance and reliability of tests for specific purposes, is the development and dissemination of alternative methods and approaches, their application in industry and their acceptance by regulators[44]. Criteria and processes for test method validation have been developed and implemented also in the US (through the Interagency Coordinating Committee on the Validation of Alternative Methods, or ICCVAM), Japan (through the Japanese Centre for the Validation of Alternative Methods or JaCVAM), and internationally through the OECD (The Organisation for Economic Co-operation and Development)[45].

EPA developed the Endocrine Disruptor Screening Program, a two-tiered screening and testing process, to determine whether certain substances may have a potential affect on estrogen, androgen and thyroid hormone systems[46]. Tier 1 screening (T1S) implies the use of assays sensitive enough to detect potential EDCs, whereas issues

of dose–response, relevance of the route of exposure, sensitive life stages and other information for hazard characterization would be resolved in the Tier 2 testing phase[10]. Because T1S is less expensive and time consuming than Tier 2 testing, equivocal effects in T1S could be replicated or evaluated further in additional short-term assays before more extensive Tier 2 testing is initiated.

Although the validation of toxicological testing methods for EDCs end points is well underway, much controversy remains over the lack of reproducibility of observed low dose effects[47].

The dawning of the new age of toxicology

Since 1986 the European Union has invested some $300 million on the development and validation of alternative approaches. A new initiative called Safety Evaluation Ultimately Replacing Animal Testing (SEURAT), the largest EU research initiative ever on alternative methods to animal testing, co-funded by the European Commission and Cosmetics Europe (a European Union Seventh Framework Programme (FP7/2007-2013)) have dedicated another 50 million euro to investing animal-free methods to long-term toxicity endpoints.

In 2007, the United States National Academy of Sciences (NAS) published *Toxicity Testing in the 21st Century – a Vision and a Strategy*, a report which envisioned a new approach to toxicology. In the past animal experiments used to be the most important technology and the new vision turns the traditional procedures upside down[30]. The vision takes its starting point from the presumption that most toxicants act by interfering with pivotal cellular structures and regulatory pathways. It is presumed that knowledge of these pathways and knowledge of the action of toxicants on these pathways would allow predictions of toxicity on the level of the whole organism. The procedure would begin with *in vitro* or *in silico* approaches based on human material to define the affected pathways[30]. To fill remaining gaps of knowledge, limited testing in animals would then be performed as an additional step. Scientists will be able to predict toxicity based upon an in-depth knowledge of the sequence of events at the cellular, subcellular and molecular levels in specific metabolic pathways and reduce the time, money and animals heretofore required[41]. The future will bring higher throughput assays, better systems biology modelling, better integration of data from omics technologies and better cell sources[5]. This means nothing less than changing toxicology from being a predominantly observational craft and regulatory support discipline back to a natural science with all its dimensions.

References

1. Benachour, N., Clair, E., Mesnage, R., Séralini,G.-E. (2012) Endocrine disruptors: New discoveries and possible progress of evaluation. In: *Advances in Medicine and Biology Vol. 29* (Berhardt, L. V., ed.), Nova Science Publishers, Inc., Hauppauge NY, pp 1-57.
2. McLachlan, J. A. (2001) Environmental signaling: What embryos and evolution teach us about endocrine disrupting chemicals. *Endocr. Rev.* **22**, 319-341.
3. Frye, C., Bo, E., Calamandrei, G., Calzà, L., Dessì-Fulgheri, F., Fernández, M., Fusani, L., Kah, O., Kajta, M., Le Page, Y., Patisaul, H. B., Venerosi, A., Wojtowicz, A. K., Panzica, G. C. (2012) Endocrine disruptors: a review of some sources, effects, and mechanisms of action on behavior and neuroendocrine systems. *J. Neuroendocrinol.* **24**, 144-159.
4. Waring, R. H., Harris, R. M. (2005) Endocrine disrupters: a human risk? *Mol. Cell. Endocrinol.* **244**, 2-9.
5. Leist, M., Lidbury, B. A., Yang, C., Hayden, P. J., Kelm, J. M., Ringeissen, S., Detroyer, A., Meunier, J. R., Rathman, J. F., Jackson, G. R. Jr., Stolper, G., Hasiwa, N. (2012a) Novel technologies and an overall strategy to allow hazard assessment and risk prediction of chemicals, cosmetics, and drugs with animal-free methods. *ALTEX* **29**, 373-388.
6. Diamanti-Kandarakis, E., Bourguignon, J-P., Giudice, L. C., Hauser, R., Prins, G. S., Soto, A. M., Zoeller, R. T., Gore, A. C. (2009) Endocrine-disrupting chemicals: an endocrine society scientific statement. *Endocr. Rev.* **30**, 293-342.
7. SPH (2003) Endocrine disruptors. SPH – School of Public Health, University of Minnesota. Available at http://enhs.umn.edu/current/5103/endocrine/character.html, downloaded July 2, 2013.
8. European Commission (2012) Endocrine Disruptors Website. Available at http://ec.europa.eu/environment/endocrine/definitions/endodis_en.htm, downloaded July 3, 2013.
9. Mendes, J. J. A. (2002) The endocrine disrupters: a major medical challenge. *Food Chem. Toxicol.* **40**, 781-788.
10. Hotchkiss, A. K., Rider, C. V., Blystone, C. R., Wilson, V. S., Hartig, P. C., Ankley, G. T., Foster, P. M., Gray, C. L., Gray, L. E. (2008) Fifteen years after „Wingspread" – environmental endocrine disrupters and human and wildlife health: where we are today and where we need to go. *Toxicol. Sci.* **105**, 235-259.
11. WHO (2013) IPCS International Programme on Chemical Safety, Global assessment of the state-of-the-science of endocrine disruptors: Chapter 6: Exposure of Selected Potential EDCs in Humans and Wildlife. WHO – World Health Organization, Geneva. Available at http://www.who.int/ipcs/publications/new_issues/endocrine_disruptors/en/, downloaded July 2, 2013.
12. Kmetič, I., Murati, T., Kvakan, K., Ivanjko, M., Šimić, B. (2012) Poliklorirani bifenili – toksičnost i rizici. *Croat. J. Food Sci. Technol.* **4**, 71-80.
13. Kidd, K. A., Becher, G., Bergman, Å. Muir, D. C. G., Woodruff, T. J. (2013) Human and wildlife exposures to EDCs. In: *State of the Science of Endocrine Disrupting Chemicals – 2012* (Bergman, Å., Heindel, J. J., Jobling, S., Kidd, K. A., Zoeller, R. T., eds.), WHO Press, World Health Organization, Geneva, pp 189-250.
14. Corsolini, S. (2009) Industrial contaminants in Antarctic biota. *J. Chromatogr. A* **1216**, 598-612.
15. Arukwe, A. (2001) Cellular and molecular responses to endocrine-modulators and the impact on fish reproduction. *Mar. Pollut. Bull.* **42**, 643-655.
16. Kniewald, J., Mildner, P., Kniewald, Z. (1979) Effects of *s*-triazine herbicides on hormone-receptor complex formation, 5 alpha-reductase and 3 alpha-hydroxysteroid dehydrogenase activity at the anterior pituitary level. *J. Steroid Biochem.* **11**, 833-838.

17. Wuttke, W., Jarry, H., Seidlova-Wuttke, D. (2010) Definition, classification and mechanisms of action of endocrine disrupting chemicals. *Hormones* **9**, 9-15.

18. Kniewald, Z., Massa, R., Motta, M., Martini, L. (1971) Feedback mechanisms and the control of the hypothalamohypophysial complex. In: *Steroid Hormones and Brain Function.* Proceedings of a Conference held May 1970, University of California, Los Angeles (Sawyer, C. H., Gorski, R. A., eds.), California Press, pp 289-299.

19. Sanderson, J. T. (2006) The steroid hormone biosynthesis pathway as a target for endocrine-disrupting chemicals. *Toxicol. Sci.* **94**, 3-21.

20. Waring, R. H., Harris, R. M. (2011) Endocrine disrupters – A threath to women's health? *Maturitas* **68**, 111-115.

21. Hayes, T. B, Anderson, L. L, Beasley, V. R, de Soll,a S. R, Iguchi, T., Ingraham, H., Kestemont, P., Kniewald, J., Kniewald, Z., Langlois, V. S., Luque, E. H., McCoy, K. A., Muñoz-de-Toro, M., Oka, T., Oliveira, C. A., Orton, F., Ruby, S., Suzawa, M., Tavera-Mendoza, L. E., Trudeau, V. L., Victor-Costa, A. B., Willingham, E. (2011) Demasculinization and feminization of male gonads by atrazine: consistent effects across vertebrate classes. *J. Steroid Biochem.* **127**, 64-73.

22. Rudel, R. A., Perovich, L. J. (2009) Endocrine disrupting chemicals in indoor and outdoor air. *Atmos. Environ*. **43**, 170-181.

23. Kmetič, I., Radošević, K., Murati, T., Šimić, B., Kniewald, Z., Kniewald, J. (2009) Lindane-induced cytotoxicity and the role of vitamin E in *Chinese hamster ovary* (CHO-K1) cells. *Toxicol. Mech. Method.* **19**, 518-523.

24. Kniewald, J., Peruzović, M, Gojmerac, T., Milković, K., Kniewald, Z. (1987) Indirect influence of *s*-triazines on rat gonadotropic mechanism at early postnatal period. *J. Steroid Biochem.* **27**, 1095-1100.

25. Newbold, R. R. (2004) Lessons learned from perinatal exposure to diethylstilbestrol. *Toxicol. Appl. Pharm.* **199**, 142-150.

26. Mori, C., Todaka, E. (2011) *Environmental Contaminants and Children's Health: Sustainable Health Science for Future Generations*, Maruzen Planet Co., Ltd., Tokyo.

27. Harris, R. M., Waring, R. H. (2012) Diethylstilboestrol – a long-term legacy. *Maturitas* **72**, 108-112.

28. Sonnenschein, C., Soto, A. M. (1998) An updated review of environmental estrogen and androgen mimics and antagonists. *J. Steroid Biochem.* **65**, 143-150.

29. Beard, J. (2006) DDT and human health. *Sci. Total. Environ.* **355**, 78-89.

30. Leist, M., Hartung, T., Nicotera, P. (2008) The dawning of a new age of toxicology. *ALTEX* **25**, 103-114.

31. European Commission (2013a) REACH. Available at http://ec.europa.eu/environment/chemicals/reach/reach_intro.htm, downloaded July 11, 2013.

32. NC3Rs (2013) What are the 3Rs?. NC3Rs – National Centre for the Replacement, Refinement & Reduction of Animals in Research. Available at http://www.nc3rs.org.uk/page.asp?id=7, downloaded July 9, 2013.

33. Zurlo, J., Rudicille, D., Goldberg, A. M. (1996) The three r's: The way forward. *Environ. Health Persp.* **104**, 878 – 880.

34. Leenaars, M., Hooijmans, C. R., van Veggel, N., ter Riet, G., Leeflang, M., Hooft, L., van der Wilt, G. J., Tillema, A., Ritskes-Hoitinga, M. (2012) A step-by-step guide to systematically identify all relevant animal studies. *Lab. Anim.* **46**, 24-31.

35. Kniewald, J., Kmetič, I., Gaurina-Srček, V., Kniewald, Z. (2005) Alternative models for toxicity testing of xenobiotics. *Arh. Hig. Rada Toksikol.* **56**, 195-204.

36. Bhanushali, M., Bagale, V., Shirode, A., Joshi, Y., Kadam, V. (2010) An *in-vitro* toxicity testing – a reliable alternative to toxicity testing by reduction, replacement and refinement of animals. *International Journal of Advances in Pharmaceutical Sciences* **1**, 15-31.

37. Aardema, M. J., MacGregor, J. T. (2002) Toxicology and genetic toxicology in the new era of „toxicogenomics": impact of „-omics" technologies. *Mutat. Res.* **499**, 13-25.

38. AltTox.org (2013a) – Omics, bioinformatics & computational biology. Available at http://alttox.org/ttrc/emerging-technologies/-omics, downloaded July 5, 2013.

39. Basketter, D. A., Clewell, H., Kimber, I., Rossi, A., Blaauboer, B., Burrier, R., Daneshian, M., Eskes, C., Goldberg, A., Hasiwa, N., Hoffmann, S., Jaworska, J., Knudsen, T. B., Landsiedel, R., Leist, M., Locke, P., Maxwell, G., McKim, J., McVey, E. A., Ouédraogo, G., Patlewicz, G., Pelkonen, O., Roggen, E., Rovida, C., Ruhdel, I., Schwarz, M.,Schepky, A., Schoeters, G., Skinner, N., Trentz, K., Turner, M., Vanparys, P., Yager, J., Zurlo, J., Hartung, T. (2012) T[4] Report: A roadmap for the development of alternative (non-animal) methods for systemic toxicity testing. *ALTEX* **29**, 3-91.

40. Houck, K. A., Kavlock, R. J. (2008) Understanding mechanisms of toxicity: Insights from drug discovery research. *Toxicol. Appl. Pharm.* **277**, 163-178.

41. Wilcox, N., Goldberg, A. (2011) Food for thought ... on validation. A puzzle or a mystery: an approach founded on new science. *ALTEX* **28**, 3-8.

42. Balls, M., Fentem, J. H. (1999) The validation and acceptance of alternatives to animal testing. *Toxicol. in Vitro* **13**, 837-846.

43. Leist, M., Hasiwa, N., Daneshian, M., Hartung, T. (2012b) Validation and quality control of replacement alternatives – current status and future challenges. *Toxicol. Res.* **1**, 8-22.

44. European Commission (2013b) European Union Reference Laboratory for alternatives to animal testing (EURL ECVAM). Available at http://ihcp.jrc.ec.europa.eu/our_labs/eurl-ecvam, downloaded July 19, 2013.

45. AltTox.org (2013b) Toxicity testing overview. Available at http://alttox.org/ttrc/tox-test-overview/, downloaded June 29, 2013.

46. US EPA (2013) Endocrine Disruptor Screening Program (EDSP). US EPA – United States Environmental Protection Agency. Available at http://www.epa.gov/endo/, downloaded September 4, 2013.

47. Martin, O. V., Lester, J. N., Voulvoulis, N., Boobis, A. R. (2007) Human health and endocrine disruption: a simple multicriteria framework for the quantitative assessment of end point-specific risks in a context of scientific uncertainty. *Toxicol. Sci*. **98**, 332-347.

Toxicological Safety Assessment and Antioxidant Activity of Sodium Copper Chlorophyllin

Goran Gajski[1*], Ivana Novak[2], Ana-Marija Domijan[3], Marko Gerić[1] and Vera Garaj-Vrhovac[1]

Scietific Note

[1]Mutagenesis Unit, Institute for Medical Research and Occupational Health, Zagreb, Croatia

[2]Toxicology Unit, Institute for Medical Research and Occupational Health, Zagreb, Croatia

[3]Department of Pharmaceutical Botany, Faculty of Pharmacy and Biochemistry, University of Zagreb, Zagreb, Croatia

Present study aimed to investigate the toxicological profile of chlorophyllin (CHL) as well as its free radical scavenging capacity. Toxicological profile of CHL was assessed by measuring its cyto/genotoxic potential in human peripheral blood lymphocytes (HPBLs) after treatment with different concentrations (0.1-100 μg/ml) for 4 and 24 h. After the treatment, cytotoxicity and genotoxity was measured using a battery of bioassays. Results of cytotoxicity showed that CHL in tested concentrations had no effect on HPBLs viability. Additionally, CHL in concentration range tested did not cause DNA damage that could be detected with the comet assay and did not induce statistically significant difference in either micronucleus assay parameter. The antioxidant activity of CHL was determined using the DPPH assay. Results of DPPH assay revealed that CHL is an effective DPPH· scavenger with the concentration-dependent radical scavenging ability. The antioxidant activity of CHL when compared to some of well-known and powerful antioxidants, such as Trolox, ascorbic acid and butylated hydroxytoluene (BHT) was about 1.6 and 1.9 fold lower than that of ascorbic acid and Trolox, respectively, and 4.3 fold higher than that of BHT. Taken together, we have shown that CHL has no impact on cell viability and genome damage in the concentration range tested and therefore it can be considered safe from the aspect of cyto/genotoxicity. Since CHL also displayed radical scavenging ability it could be a perfect candidate for testing as a novel antioxidant.

Key words:

Chlorophyllin, human lymphocytes, cytotoxicity, genotoxicity, antioxidant activity

[*]Corresponding author: ggajski@imi.hr

Introduction

Chlorophyllins are derivatives of chlorophyll in which the central magnesium atom is replaced by other metals, such as cobalt, copper or iron. Sodium copper chlorophyllin (CHL) is a semi-synthetic mixture of water-soluble sodium copper salts derived from chlorophyll that is widely used as a colour additive in foods, drugs, cosmetics and as a dietary supplement (1,2). Chemical structure of CHL is shown in Figure 1. Although both chlorophyll and CHL are allowed as food colorants in Europe (3) and Brazil (4), CHL is permitted only in one type of citrus drink in the United States of America (5).

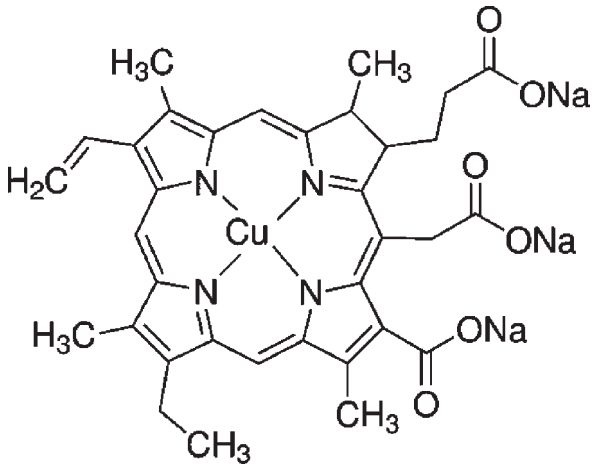


Fig. 1 – Chemical structure of chlorophyllin sodium copper salt

It is shown that CHL has beneficial effects on human health (2). Some studies indicate that this can be due to its anti-oxidative properties (2,6). *In vivo* and *in vitro* studies demonstrate that CHL has potential to inhibit lipid peroxidation (oxidative damage of lipids) and prevent oxidative DNA damage (7,8,9,10,11). Importantly, the antioxidant activity of CHL was found to be much higher than of natural chlorophylls (12,13). Also, in a large number of studies it is found that CHL exhibits anti-mutagenic and anti-carcinogenic activities against many dietary and environmental agents (1,2). Those studies revealed that anti-mutagenic and anti-carcinogenic properties of CHL may be attributed to ability of CHL to bind carcinogen by forming tight complex, and thus diminish its bioavailability (2,6,14,15).

The aim of the present study was to investigate impact of CHL on cell and genome damage in order to assess its safety from the aspect of cyto/genotoxicity. This was

done on primary human peripheral blood lymphocytes (HPBLs) as they are sensitive biomarkers of exposure. Cytotoxic effect was explored by differential staining of the HPBLs after the treatment with CHL using fluorescence microscope. DNA damage was measured with the alkaline comet assay while further genotoxic activity of CHL was assessed with the micronucleus (MN) assay. Moreover, to evaluate its antioxidant potential, free radical scavenging capacity of CHL was determined with the DPPH assay. DPPH is a well-known scavenger of radicals. Therefore, rate reduction of a chemical reaction upon addition of DPPH is used as an indicator of the radical nature of that reaction. Hence, scavenging of DPPH free radical is the basis of a common antioxidant assay (16,17).

Materials and methods

Chemicals

CHL, acridine orange (AO), cytochalasin-B, disodium EDTA, ethidium bromide (EtBr), Histopaque, low melting point (LMP) and normal melting point (NMP) agaroses, RPMI 1640 medium, Triton X-100, L-ascorbic acid, di(phenyl)-(2,4,6-trinitrophenyl)iminoazanium (2,2-diphenyl-1-picrylhydrazyl, DPPH), (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) and buthylated hydroxytoluene (BHT) were purchased from Sigma (St Louis, USA). Giemsa solution was from Merck (Darmstadt, Germany) and Chromosome kit P was from Euroclone (Milano, Italy). All other used chemicals were laboratory-grade and were purchased from Kemika (Zagreb, Croatia).

Blood sampling and treatment

Toxicological characterization of CHL was evaluated on HPBLs obtained from a healthy female, non-smoking donor. Subject gave informed consent to participate in this study. The study was approved by the institutional ethics committee and observed the ethical principles of the Declaration of Helsinki. The donor has not been exposed to ionizing radiation or to known genotoxic chemicals that might have interfered with the results of the testing for a year before blood sampling. Blood was drawn by antecubital venipuncture into heparinized vacutainers containing lithium heparin as anticoagulant under aseptic conditions. Blood samples were treated with different concentrations of CHL (0.1-100 μg/ml) for 4 and 24 h. In each experiment, a non treated control was included.

Cytotoxicity assay

Cell viability was determined by differential staining with AO and EtBr on fluorescence microscope (18). HPBLs were isolated by Histopaque density centrifugation method (19). The slides were prepared using 200 µl of HPBLs and 2 µl of stain. A total of 100 cells per repetition were examined with an Olympus BX-51 microscope (Tokyo, Japan), at 400× magnification. Cells were classified as follows: live cells with functional membrane with uniform green staining of the nucleus, and necrotic cells with uniform red staining of the nucleus.

Comet assay

The alkaline comet assay was carried out as described by Singh et al. (20) with minor modifications (21). After the exposure, whole blood was mixed with 0.5% LMP agarose and added to fully frosted slides pre-coated with 0.6% NMP agarose. After solidified the slides were covered with 0.5% LMP agarose, and lysed (2.5 mol/dm^3 NaCl, 100 mmol/dm^3 Na_2EDTA, 10 mmol/dm^3 Tris, 1% sodium sarcosinate, 1% Triton X-100, 10% dimethyl sulfoxide; pH 10) over night at 4°C. After the lysis the slides were placed into alkaline solution (300 mmol/dm^3 NaOH, 1 mmol/dm^3 Na_2EDTA; pH 13) for 20 min at 4°C to allow DNA unwinding and subsequently electrophoresed for 20 min at 1 V/cm. Finally, the slides were neutralized in 0.4 mol/dm^3 Tris buffer (pH 7.5), stained with EtBr and analyzed at 250× magnification using an epifluorescence microscope (Zeiss, Göttingen, Germany) connected through a camera to an image analysis system (Comet Assay II; Perceptive Instruments Ltd., Haverhill, Suffolk, UK). The level of DNA damage was expressed as percentage of DNA in the tail, and a total of 100 randomly captured comets were examined from each slide.

Micronucleus assay

The MN assay was performed according to the guidelines of Fenech and Morley (22) with minor modifications (21). After the exposure, whole blood was incubated in a Euroclone medium at 37ºC in an atmosphere of 5% CO_2. Cytochalasin-B was added at a final concentration of 3 µg/ml, 44 h after the culture was started. The cultures were harvested after 72 h. The lymphocytes were fixed in methanol-acetic acid, air-dried and stained with Giemsa solution. The binuclear HPBLs were analyzed under a light microscope (Olympus CX41, Tokyo, Japan) at 400× magnification. Micronuclei (MNi), micronucleated cells (MNed), nucleoplasmic bridges (NPBs) and nuclear buds (NBUDs) were counted in 1000 binucleated cells and were scored according to the HUMN project criteria published by Fenech et al. (23).

DPPH assay

The antioxidant activity of CHL was evaluated according to the DPPH radical scavenging method described by Šeruga et al. (24). Briefly, 50 µl of aqueous CHL solution in different concentrations (1.0-30 µmol/dm^3) were mixed with 120 µl of DPPH · solution (1 mmol/dm^3, in methanol) and 1880 µl of methanol. The reaction mixture was incubated in the dark at room temperature for 15 min and then the absorbance of the mixture (A_{sample}) was measured at 517 nm against the blank sample (blank sample: 50 µl of CHL solution and 2000 µl of methanol). Absorbance of DPPH · solution in the absence of CHL was measured as control (A_{DPPH}). Inhibition of free radical DPPH · was calculated for different concentrations of CHL according to the following equation: % inhibition = $[(A_{DPPH\cdot} - A_{sample}) / A_{DPPH}] \times 100$. In order to obtain the EC_{50} index, defined as the amount of antioxidant (µmol/dm^3) needed to reduce the initial concentration of DPPH · by 50 %, different concentrations of CHL in the reaction mixture were plotted against calculated percentage inhibition of DPPH ·. As reference compounds BHT, Trolox and ascorbic acid were used. All tests were carried out in triplicate.

Statistics

Statistical analysis was done by use of the Statistica 5.0 software package. For the comet assay multiple comparisons between groups were done by means of ANOVA on log-transformed data. Post hoc analyses of differences were done by using the Scheffé test. As for the cell viability, statistical significance was analyzed using the Student's *t*-test. The significance of the MN assay parameters was tested using the χ^2-test. Probability values of $P<0.05$ were considered statistically significant.

Results

Toxicological profile of chlorophyllin

Viability of the cells was not affected with concentration of CHL as high as 100 µg/ml and was above 98.77±3.42% and 94.61±2.49% after 4 and 24 h treatment, respectively (Figure 2A). Reduction of cell viability was less than 10%, and therefore selected concentrations were used for further genotoxic experiments. DNA damage in HPBLs was determined with the alkaline comet assay. No statistically significant difference was noted in percentage of tail DNA in cells treated with CHL compared to untreated control (Figure 2B). The genotoxic activity of CHL was further evaluated using the MN assay. The induction of MNi, MNed, NPBs and NBUDs was assessed in binucleated

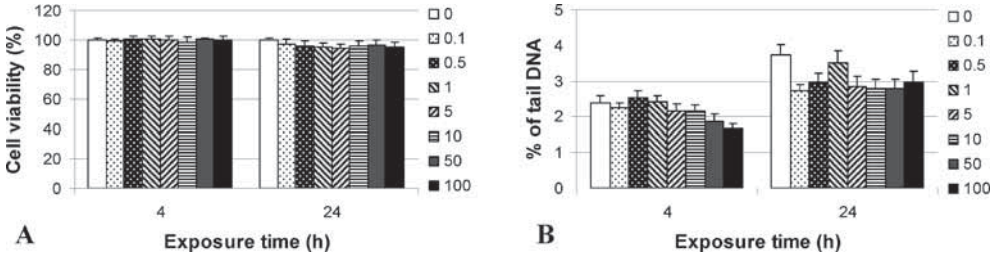


Fig. 2 – The effects of chlorophyllin (CHL) on the viability and DNA damage in human peripheral blood lymphocytes (HPBLs). After the exposure to different concentrations of CHL (0.1-100 μg/ml) for 4 and 24 h (A) the cell viability was determined by differential staining with acridine orange (AO) and ethidium bromide (EtBr); and (B) DNA damage (expressed as percentage of tail DNA) was assessed with the alkaline comet assay. Note: there were no statistically significant differences between treated samples compared to corresponding control ($P<0.05$)

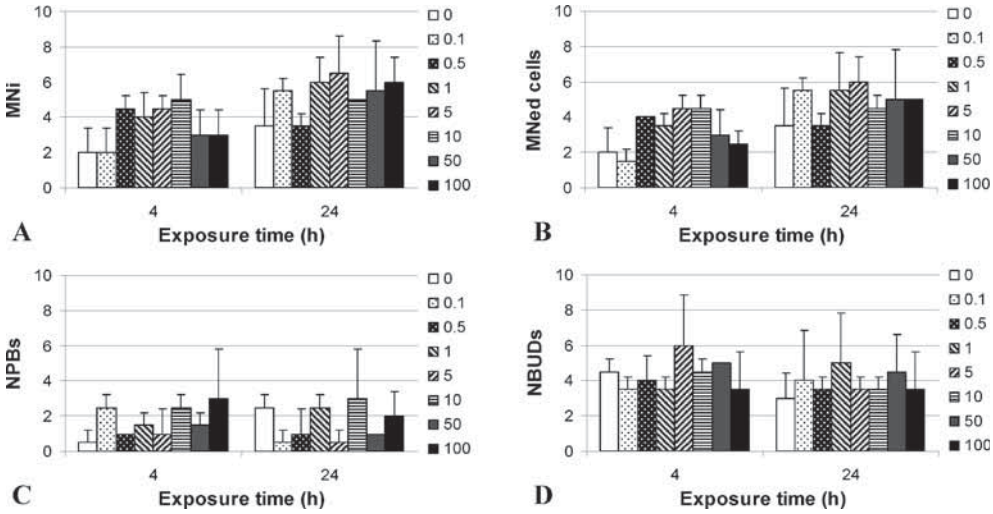


Fig. 3 – The effects of chlorophyllin (CHL) on the micronucleus (MN) assay parameters in human peripheral blood lymphocytes (HPBLs). Number of (A) micronuclei, MNi; (B) micronucleated cells, MNed; (C) nucleoplasmic bridges, NPBs; and (D) nuclear buds, NBUDs in binucleated HPBLs exposed to CHL (0.1-100 μg/ml) for 4 and 24 h. Incidence of MNi, MNed, NPBs and NBUDs was evaluated by analyzing 1000 binucleated cells. Note: there were no statistically significant differences between treated samples compared to corresponding control ($P<0.05$)

HBBLs. No statistically significant difference was found in either MN assay parameter in cells treated with CHL compared to untreated control (Figure 3).

Antioxidant activity of chlorophyllin

Results of the DPPH assay showed that CHL is an effective DPPH · scavenger. As can be seen from Figure 4, radical scavenging ability of CHL was concentration-dependent, and was linear in the concentration range from 1.0 μmol/dm^3 to 30 μmol/

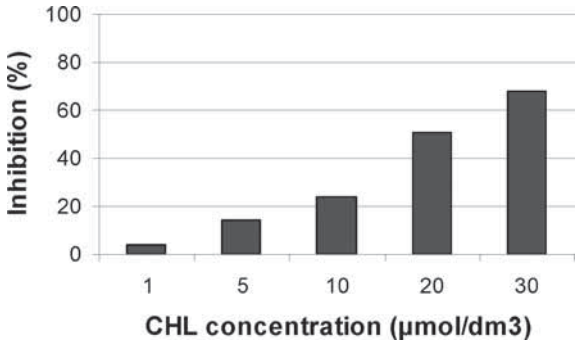


Fig. 4 – The antioxidant activity of chlorophyllin (CHL) assessed with the DPPH assay. The antioxidant activity/DPPH free radical scavenging activity of CHL in concentration range (1.0-30 µmol/dm^3) was concentration-dependent

dm^3 (r = 0.994). From the linear regression curve calculated EC_{50} value of CHL (concentration of CHL that reduce DPPH · activity by 50%) was 21.06 µmol/dm^3. By comparing the EC_{50} value of CHL with Trolox, ascorbic acid and BHT, the DPPH · free radical scavenging capacity of CHL was about 1.6 and 1.9 fold lower than of ascorbic acid and Trolox, respectively, and 4.3 fold higher than of BHT (Table 1). The antioxidant activity of CHL and reference compounds decreased in the order: ascorbic acid > Trolox > CHL > BHT.

Table 1 – Values of EC_{50} index of different antioxidants, ascorbic acid, Trolox and buthylated hydroxytoluene (BHT) as reference compounds, compared to chlorophyllin (CHL) assessed with the DPPH assay

Antioxidant	**EC_{50} (µmol/dm^3)**
Ascorbic acid	11.06
Trolox	13.40
CHL	21.06
BHT	91.23

Discussion

CHL is well known for its anti-mutagenic and anti-carcinogenic properties, however there are some studies reporting its tumour-enhancing and genotoxic effects. These contradictory results are attributed to variability in the chemical composition of the preparations of CHL used, concentration and source of CHL, as well as to experimental model (2,25).

In that manner, present study aimed to investigate toxicological profile of CHL on HPBLs as sensitive biomarkers of exposure. In our study we explored the possible cyto/genotoxic effect of CHL using cytotoxicity assay, comet assay and MN assay to evaluate the impact of CHL on cell and genome damage (23,26). Additionally, to assess possible antioxidant activity of CHL we used DPPH assay. Results showed that CHL in concentration range tested (0.1-100 μg/ml) was not cyto/genotoxic to HPBLs. Moreover, results of the DPPH assay confirmed that CHL has a marked capacity to scavenge DPPH · free radical indicating that CHL acts as a free radical scavenger probably due to its facile electron-donating ability.

These results are consistent with earlier studies showing that CHL has no impact on DNA damage. CHL did not induce DNA damage detected with the comet assay in HEp-2 cells (27) and was not genotoxic to V79 cells evaluated by the MN assay (28). Pietrzak et al. (29) observed that exposure of HL-60 cells to CHL had no significant effect on expression of phosphorylated H2AX (γH2AX) that is considered to be a marker of DNA damage (30,31). Our results are in agreement with Ferruzzi et al. (12) and Lanfer-Marquez et al. (13) who also suggested that CHL may act as an electron-donating antioxidant in addition to other studies that confirmed antioxidant activity of CHL (9,11,32).

Conclusions

Present study provides evidence that CHL has no impact on cell viability and genome damage in the concentration range tested; hence it is to be presumed that CHL is safe from the aspect of cyto/genotoxicity. Since CHL also displayed radical scavenging ability it could be a perfect candidate for testing as a novel antioxidant. Since there is wide variation in the health-related biological activities of CHL that may arise from differences in the degree of purity and composition of the commercial grade CHL preparations there is a need for the standardisation of CHL and for the development of a simplified method for the characterization of CHL preparations. Further investigations are also needed to determine the most effective CHL dose ranges as well as the existence of possible adverse effects, if any.

Acknowledgments

This work was supported by funds of the Ministry of Science, Education and Sport of the Republic of Croatia (Projects Nos. 022-0222148-2125, 022-0222148-2142).

References

1. Sarkar D, Sharma A, Talukder G. Chlorophyll and chlorophyllin as modifiers of genotoxic effects. *Mutat Res* 1994; 318: 239-247.
2. Tumolo T, Lanfer-Marquez UM. Copper chlorophyllin: a food colorant with bioactive properties? *Food Res Int* 2012; 46: 451-459
3. European Commission (EC). Commission Directive 94/36/EC. *Official Journal of the European Communities, L237,* 1994; 13-29.
4. Agência Nacional de Vigilância Sanitária (ANVS). *Regulamento Técnico sobre atribuição de aditivos e seus limites máximos para alimentos*: Agência Nacional de Vigilância Sanitária – Brasil Resolução RDC 64 de 16 de setembro de 2008, 2008.
5. US Food and Drug Administration (FDA). *Listing of color additives exempt fromcertification: Sodium copper chlorophyllin.* US Food and Drug Administration, Department of Health and Human Services Federal Register 67FR49567/67FR35429, 2002.
6. Ferruzzi MG, Blakeslee J. Digestion, absorption, and cancer preventive activity of dietary chlorophyll derivatives. *Nutr Res* 2007; 27: 1-12.
7. Sato M, Konagai K, Kimura R, Murata T. Effect of sodium copper chlorophyllin in lipid peroxidation. V. Effect on peroxidation damage on rat liver lysosomes. *Chem Pharm Bull* 1983; 31: 3665-3670.
8. Sato M, Konagai K, Kuwana T, K, Kimura R, Murata T. Effect of sodium copper chlorophyllin in lipid peroxidation. VII. Effect of its administration on the stability of rat liver lysosomes. *Chem Pharm Bull* 1984; 32: 2855-2858.
9. Boloor KK, Kamat JP, Devasagayam TPA. Chlorophyllin as a protector of mitochondrial membranes against γ-radiation and photosensitization. *Toxicology* 2000; 155: 63-71.
10. Kumar SS, Shankar B, Sainis KB. Effect of chlorophyllin against oxidative stress in splenic lymphocytes in vitro and in vivo. *Bioch Biophys Acta* 2004; 1672: 100-111.
11. Park KK, Park JH, Jung YJ, Chung WY. Inhibitory effects of chlorophyllin, hemin and tetrakis(4-benzoic acid)porphyrin on oxidative DNA damage and mouse skin inflammation induced by 12-O-tetradecanoylphorbol-13-acetate as a possible anti-tumor promoting mechanism. *Mutat Res* 2003; 542: 89-97.
12. Ferruzzi MG, Böhm V, Courtney PD, Schwartz SJ. Antioxidant and antimutagenic activity of dietary chlorophyll derivatives determined by radical scavenging and bacterial reverse mutagenesis assays. *J Food Sci* 2002; 67: 2589-2595.
13. Lanfer-Marquez UM, Barros RMC, Sinnecker P. Antioxidant activity of chlorophylls and their derivatives. *Food Res Int* 2005; 38: 885-891.
14. Egner PA, Muñoz A, Kensler TW. Chemoprevention with chlorophyllin in individuals exposed to dietary aflatoxin. *Mutat Res* 2003; 523-524: 209-216.
15. Pratt MM, Reddy AP, Hendricks JD, Pereira C, Kensler TW, Bailey GS. The importance of carcinogen dose in chemoprevention studies:quantitative interrelationships between, dibenzo[a, l] pyrene dose, chlorophyllin dose, target organ DNA adduct biomarcers and final tumor outcome. *Carcinogenesis* 2007; 28: 611-624.
16. Sharma OP, Bhat TK. DPPH antioxidant assay revisited. Food Chem 2009; 113: 1202-1205.
17. Gülçin İ. Antioxidant activity of food constituents: an overview. Arch Toxicol 2012; 86: 345-391.
18. Duke RC, Cohen JJ. Morphological and biochemical assays of apoptosis. In: Coligan JE, Kruisbeal AM, (Eds.), Current protocols in immunology. New York: John Willey & Sons 1992; 1-3.

19. Singh NP. Microgels for estimation of DNA strand breaks, DNA protein crosslinks and apoptosis. *Mutat Res* 2000; 455: 111-127.

20. Singh NP, McCoy MT, Tice RR, Schneider LL. A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp Cell Res* 1988; 175: 184-191.

21. Gajski G, Garaj-Vrhovac V, Oreščanin V. Cytogenetic status and oxidative DNA-damage induced by atorvastatin in human peripheral blood lymphocytes: standard and Fpg-modified comet assay. *Toxicol Appl Pharmacol* 2008; 231: 85-93.

22. Fenech M, Morley AA. Measurement of micronuclei in lymphocytes. *Mutat Res* 1985; 147: 29-36.

23. Fenech M, Chang WP, Kirsch-Volders M, Holland N, Bonassi S, Zeiger E. HUMN project: detailed description of scoring criteria for the cytokinesis-block micronucleus assay using isolated human lymphocyte cultures. *Mutat Res* 2003; 534: 65-75.

24. Šeruga M, Novak I, Jakobek L. Determination of polyphenols content and antioxidant activity of some red wines by differential pulse voltammetry, HPLC and spectrophotometric methods. *Food Chem* 2011; 124: 1208-1216.

25. Chernomorsky S, Rancourt R, Virdi K, Segelman A, Poretz RD. Antimutagenicity, cytotoxicity and composition of chlorophyllin copper complex. *Cancer Lett* 1997; 120: 141-147.

26. Collins AR, Oscoz AA, Brunborg G, Gaivão I, Giovannelli L, Kruszewski M, Smith CC, Stetina R. The comet assay: topical issues. *Mutagenesis* 2008; 23: 143-151.

27. Botelho MV, Orlandi JM, de Melo FL, Mantovani MS, Linhares RE, Nozawa C. Chlorophyllin protects HEp-2 cells from nuclear fragmentation induced by poliovirus. *Lett Appl Microbiol* 2004; 39: 174-177.

28. Bez GC, Jordão BQ, Vicentini VE, Mantovani MS. Investigation of genotoxic and antigenotoxic activities of chlorophylls and chlorophyllin in cultured V79 cells. *Mutat Res* 2001; 497: 139-145.

29. Pietrzak M, Halicka HD, Wieczorek Z, Wieczorek J, Darzynkiewicz Z. Attenuation of acridine mutagen ICR-191 – DNA interactions and DNA damage by the mutagen interceptor chlorophyllin. *Biophys Chem* 2008; 135: 69-75.

30. Rogakou EP, Pilch DR, Orr AH, Ivanova VS, Bonner WM. DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139. *J Biol Chem* 1998; 273: 5858-5868.

31. Sedelnikova OA, Rogakou EP, Panyutin IG, Bonner WM. Quantitative detection of (125)IdU-induced DNA double-strand breaks with gamma-H2AX antibody. *Radiat Res* 2002; 158: 486-492.

32. Kamat JP, Boloor KK, Devasagayam TP. Chlorophyllin as an effective antioxidant against membrane damage in vitro and ex vivo. *Biochim Biophys Acta* 2000; 1487: 113-127.

Aquaculture and Fish Cell Technology

Višnja Gaurina Srček*, Kristina Radošević and Zlatko Kniewald *Review*

University of Zagreb, Faculty of Food Technology and Biotechnology,
Laboratory for Cell Culture, Applications and Biotransformation,
Pierottijeva 6, HR-10000 Zagreb

Aquaculture is the fastest growing food production industry offering opportunities to increase food and nutrition security and reduce the number of unemployed persons. An important part of increasing the aquaculture production is improving the biological productivity of fish farmed species which can be achieved by modern biotechnological methods. For example, genetic improvements of growth rate, survival and feed conversion efficiency have been shown to reduce space, water and feed requirements. However, international regulatory framework for aquatic biotechnology and an adequate mechanism to facilitate technology transfer and technical assistance must be designed in order to ensure the safe and equitable use of this technology.

Fish are the most frequently used animals in the environmental risk assessment for studying the effects of different stressors on fish health and disease. Recent EU REACH regulation (Registration, Evaluation, Authorization and Restriction of Chemical substances EC 1907/2006 and 63/2010) emphasizes, when possible, the use of alternative in vitro models instead of live fish since this approach reduces the number of laboratory animals used in the toxicological studies. Accordingly, fish cells significantly contributing in assessment of chemical risk and in the implementation of the REACH regulations since serve as a test-model for studying eco toxicants and water samples for regulatory purposes, development and safety evaluation of newly substances and environmental monitoring.

Key words:

aquaculture, ecotoxicology, fish cell technology, food production, transgenesis

Oceans contain 97% of the earth's water while the other 3% is determined as freshwater. More than 70% of freshwater resources are stored in the ice and only 1% is a surface freshwater important for the human, animal and plants life, but also for the each day higher industry demands. Aquaculture involves genetic manipulation of the chosen species by keeping them captive throughout their breeding cycle, but also is source for

*Corresponding author: vgaurina@pbf.hr

fine chemicals and biopharmaceuticals (e.g. β-carotene from algae *Dunalliela salina*) which are used as food or feed additives.(Kniewald and Kniewald, 2007).

Fisheries and Aquaculture Department from the UN Food and Agriculture Organization (FAO) published in March 2013 the Global Aquaculture Production Statistics for the year 2011 (www.fao.org/fishery/topic/16140/en). World aquaculture production of food fish reached 62.7 million tonnes in 2011, up by 6.2% from 59 million tonnes in 2010. Value of farmed food fish is US$ 130 billion; while farmed aquatic algae production in 2011 was 21 million tonnes valued US$ 5.5 billion. Aquaculture contributed 40.1% to the world total fish production and almost all the seaweeds production. In 2011, the top-20 producers produced 95% of world farmed food fish. The term food fish include fishes, crustaceans, molluscs, amphibians, reptiles (excluding crocodiles) and other aquatic animals (such as sea cucumber, sea urchin, etc.) for human consumption. From the Europe, Norway is on the 7th place and Spain on the 19th. France is also important producer but dropped out from the top list of the "Best 20" after 2010, and on the 20th place entered Iran. The leader among the aquaculture producers of food fish is China with more than 38 million tonnes of annual production. The changes in the species consumption of world aquaculture during 1970 – 2011 are summarized in Table 1 and the increase index in the past period is 24.1.

Table 1 – World aquaculture production of food fish by main groups of species (million tonnes)

	1970	1980	1990	2000	2010	2011
Fin fish	1.5	2.8	8.7	20.8	38.3	41.6
Molluscs	1.1	1.8	3.6	9.8	14.2	14.4
Crustaceans	0.0	0.1	0.8	1.7	5.7	5.9
Others	0.0	0.0	0.0	0.2	0.8	0.8
Total	2.6	4.7	13.1	32.4	59.0	62.7

While the world is facing with multiple and interlinked challenges from the impacts of the on-going financial and economic crisis to greater climate changes vulnerabilities at the same time it must meet the food and nutrition needs of an expanding population up to 9 billion at this century. The fisheries and aquaculture offers opportunities to increase food and nutrition security and reduce the number of unemployed persons. Stimulated by higher demand for fish, world fisheries and aquaculture production is projected to reach 170 million tonnes in 2021, with the most of the growth coming from aquaculture. In 2010, the composition of world

aquaculture production was: freshwater fishes (56.4 %, 33.7 million tons), mollusks (23.6 %, 14.2 million tons), crustaceans (9.6 %, 5.7 million tons), diadromous fishes (6.0 %, 3.6 million tons), marine fishes (3.1 %, 1.8 million tons) and other aquatic animals (1.4 %, 814 300 tons). The number of species recorded in FAO aquaculture production statistics increased to 541 species and species groups in 2010, including 327 finfishes (5 hybrids), 102 mollusks, 62 crustaceans, 6 amphibians and reptiles, 9 aquatic invertebrates and 35 algae. Aquaculture will remain one of the fastest-growing animal food-producing sectors and employment in the fisheries and aquaculture primary sector will grow faster than in agriculture. Fish and fishery products continue to be among the most-traded food commodities worldwide therefore it is an increasing need for international cooperation for global sustainable fisheries management and biodiversity conservation. As a sector, aquaculture now makes an essential contribution to nutrition globally. It is also a growth sector, since fishing, no matter how productive, cannot meet the demand for fish, molluscs, crustaceans and algae, due to the limited natural productivity of the aquatic environment. Despite this favourable context, European aquaculture is not benefiting from this global upsurge. Therefore, on the 8th of April 2009, the European Commission adopted a strategy aimed at identifying the problems faced by European aquaculture and at putting a framework in place to promote sustainable development in the sector (http://ec.europa.eu/fisheries/documentation/videos/aquaculture/index_en.htm, reached August 27, 2013). 23 out of 28 EU countries have a coastline and the EU's coastline is 7 times as long as the US' and 4 times as long as Russia's. The EU's maritime regions are home to almost half its population and account for almost half its GDP. In terms of surface area, there is more sea than land under the jurisdiction of EU countries and including its outlying regions, the EU has the world's largest maritime territory. Croatia inside EU has better opportunities for implementation own Strategy that ever before. On the October 2012 Department of Agriculture Republic of Croatia prepared and on January 2013 published proposal of National Strategy for Fisheries Development (http://www.mps.hr/ribarstvo/UserDocsImages/NSP/NSP-ribarstvo-Nacrt.pdf, reached August 27, 2013).

Aquaculture production is vulnerable to adverse impacts of disease and environmental conditions. Disease outbreaks in recent years have affected farmed Atlantic salmon in Chile, oysters in Europe, and marine shrimp farming in several countries in Asia, South America and Africa, resulting in partial or sometimes total loss of production. In 2010, aquaculture in China suffered production losses of 1.7 million tones caused by natural disasters, diseases and pollution. Disease outbreaks virtually wiped out marine shrimp farming production in Mozambique in 2011 (The State of World Review of Fisheries and Aquaculture 2012).

As in many other research areas of the life sciences, modern biotechnology is considered to be a new tool to improve the quality and quantity of fish breed in aqua-

culture (Aerni, 2001). Finfish and shellfish can be genetically modified through gene transfer, chromosome set manipulation, interspecific hybridization, and other methods. Several useful genes have been identified and transferred into different aquatic species:

– growth hormones for increased growth and efficiency
– anti-freeze protein for increased freeze reluctance
– lysozyme for increased disease resistance
– prolactin hormones that influence hatching, osmoregulation, behavior and metabolism

Experiments with transgenic fish have shown that commercially important issues, such as enhanced growth rates, disease resistance and increased environmental tolerance can be improved. Aquabounty Farms in Waltham, MA, USA with its subsidiary Aquabounty Farms Canada in Prince Edward Island has created a salmon engineered to grow faster and to use feed more efficiently (*AquAdvantage® Salmon*). AquAdvantage® Salmon contains a gene construct consisting of a Chinook salmon growth hormone gene and a regulatory sequence for the control and expression. The expression of the introduced structural gene elicits the phenotype of enhanced growth rate and feed efficiency. The growth rate of this farmed fish can be increased by 400% to 600% while simultaneously reducing feed input by up to 25% per unit of output (http://www.aquabounty.com/products/aquadvantage-295.aspx, reached September, 16, 2013).

Fish offer a unique and little explored opportunity to be used as bio factories of pharmaceutical products and several research projects have been started in this area (Table 2). The use of transgenic fish as bio factories has been developed for the production of human insulin and factor VII by Tilapia as well as collagen and calcitonin production from other fish species (Rocha et al., 2003).

Table 2 – Research in human biopharmaceuticals in fish

Product	Species	Company
Factor VII	Tilapia	Aquagene (USA)
Insulin	Tilapia	Philippine Council for Aquatic & Marine Research & Development (USA-Canada)
Collagen	Unknown fish	Meanwhile Shida Canning Co Ltd (Japan)
Calcitonin	Salmon	DiverDrugs (Spain)

Data obtained from Rocha et al., (2003)

Contamination of aquatic environment by man-made contaminants has demanded the development of protocols and concepts for the examination of their effects on

aquatic organisms, including fish. Currently, toxicity testing on fish is mainly based on *in vivo* and these tests are designed to evaluate endpoints on mortality, lethal and sublethal effects, growth rate, reproduction and assessment of potential endocrine disruptors. OECD Guidelines for the testing of chemicals determine following tests using fish: acute toxicity for fish (Test No 203); prolonged toxicity test (Test No 204); early-life stage toxicity test (Test No 210); a short-term screening for oestrogenic and androgenic activity and aromatase inhibition (Test No 230) and fish short term reproduction assay (Test No 229). Recent EU Regulation REACH (Registration, Evaluation, Authorization and Restriction of Chemical substances EC 1907/2006 and EC 63/2010) emphasizes, when possible, the use of alternative *in vitro* models since this approach reduces the number of laboratory animals used in the toxicological studies. According to data obtained from the European Commission's sixth report on the number of experimental animals used in the 27 Member States of the European Union (EU), a total number of 1.749.178 fish were used in 2005 for experimental purposes while in 2008 this number was 1.087.155. Reduction of fish number for 37.85% could be related with changes in testing strategy using *in vitro* methods for the initial hazard evaluation of chemicals.

Animal cell lines technologies have been considered as an alternative to the use of whole animals in toxicity testing. Application of cell lines as an *in vitro* tool in the evaluation of toxicity can be an important contribution in elucidating intracellular, molecular or physiological mechanism induced by xenobiotics (Kniewald et al., 2005). In aquatic toxicology, continuous fish cell lines have been used as a tool for screening and for toxicity ranking of anthropogenic chemicals, compound mixtures and environmental samples (Fent, 2001). The first established permanent fish cell line was RTG-2 originates from gonads of juvenile rainbow trout and was developed for studying fish viruses (Wolf and Quimby, 1962). During the last four decades fish cell lines from different fish species have been established and currently about 280 cell lines from fish are developed (Lakra et al., 2010). The most frequently fish cells lines used in ecotoxicology are listed in Table 3.

Fish cell lines are mostly anchorage dependent and some of them have been adapted to grow in suspension like CHSE–*sp* cells. They grow well in most basal culture media supplemented with 5-10 % (v/v) animal serum (usually foetal bovine serum – FBS). The major limitation in the more intensive application of fish cell system is an insufficient characterisation, both with respect to their cellular and functional properties and to their culture requirements. In our Laboratory, growth and metabolic characteristics, especially the energy metabolism and its regulation have been studied in Channel Catfish Ovary (CCO) cell line. Obtained results showed that nutrient requirement of this cell line mainly depended on glutamine rather than glucose. Glutamine stimulated CCO cell growth and reduced glucose utilization whereas its deficiency in culture medium induced cell growth arrest (Slivac et al, 2008 and 2010).

Table 3 – Fish cell lines used in ecotoxicology (adapted from Bols et al., 2005)

Cell line designation	Species	Tissue of origin	Morphology	Culture collection
AB-9	Brachydanio rerio (zebrafish)	Caudal fin	Fibroblastic	ATCC CRL-2298
BB	Ictalurus nebulosus (bullhead, brown)	Connective tissue and muscle	Fibroblastic	ATCC CCL-59
BF-2	Lepomis macrochirus (bluegill)	Caudal trunk	Fibroblastic	ATCC CCL-91
CCO	Ictalurus punctatus /catfish)	Ovaries	Epithelial	ATCC CRL-2772
CHSE-214	Oncorhynchus tshawytscha (Chinook salmon)	Normal embryo	Fibroblastic	ATCC CRL-1681
EPC	Cyprinus carpio (common carp)	Epithelioma papulosum	Epithelial	ECACC-93120820
FHM	Pimephales promelas (fathead minnow)	Connective tissue and muscle	Epithelial	ATCC CCL-42
PHL	Clupeaa harengus pallasi (Pacific herring)	Larvae	Epithelial	ATCC CRL-2750
PLHC-1	Poeciliopsis lucida (topminnow) hepatocyte; liver	Hepatocellular carcinoma;	Epithelial	ATCC CRL-2406
RTG-2	Oncorhynchus mykiss (rainbow trout)	Mixed gonads	Fibroblastic	ATCC CCL-55
RTgill-W1	Oncorhynchus mykiss (rainbow trout)	Gills	Epithelial	ATCC CRL-2523
RTL-W1	Oncorhynchus mykiss (rainbow trout)	Normal liver	Epithelial	Not deposited
ZEM2S	Brachydanio rerio (zebrafish)	Embryo	Fibroblastic	ATCC CRL-2147
ZF-L	Brachydanio rerio (zebrafish)	Normal liver	Epithelial	ATCC CRL-2643

Because of ethical considerations and animal welfare, serum replacement from culture medium is demanded which required adaptation of fish cells on serum-free medium. RTG-2 and PLHC-1 cells are successfully adapted to serum-free medium (Ackermann and Fent, 1998) as well as CCO cells (Gaurina Srček et al., 2013) showing similar or better growth characteristics when compared to regular culture medium. Another disadvantage of serum use in cytotoxicity testing is its influence on toxicant availability which could be overcome by application of fish cells adapted to serum-free medium. Fish cells can be grown over a wide temperature ranges, usually bellow 30°C and temperature ranges are similar to the habitat temperature of the originating species (Fent, 2001). It has been argued that mammalian cells

that are cultured at higher temperatures and proliferate faster than fish cells may be more sensitive and better *in vitro* system for prediction of acute fish lethality when cell growth was main endpoint (Segner, 2004). Although results from several different authors (Castaño and Gómez-Lechón, 2005), as well as our own results (Radošević et al., 2011, Cvjetko et al., 2012), showed almost equal sensitivity towards cytotoxicity of tested compounds between fish and mammalian cell lines when IC50 values are compared.

Interactions of chemicals with biota take first place at a cellular level making cellular responses not only the first manifestation of toxicity but also suitable tools for the early and sensitive detection of chemical exposure. Since fish represent a most diverse group of vertebrates, with approximately 20.000 different species occupying all aquatic environments, in current test strategies fish cells represent promising test system for the assessment of chemical risks especially when taking into consideration species-specific toxic effects. Also, understanding the actions of eco toxicants on fish helps in evaluating the health of aquatic environment.

In the investigations of environmental contaminants fish-derived cell lines are being used for: determination the mechanisms by which contaminants exert their toxicity; assessing the relative toxicity of different chemical contaminants and evaluation the toxicity of environmental samples (Dayeh et al., 2003). The application of fish cells lines for toxicological and ecotoxicological purposes often means routinely evaluation common cellular responses like: cytotoxicity, cell growth, genotoxicity and xenobiotic metabolism. The cytotoxicity of chemicals can be determined by a variety of endpoints, including measures of cell death, viability and functionality, morphology, energy metabolism, cell attachment and proliferation. Change in cell viability as general response to toxicant determines fundamental cellular activities which would be expressed by many cell lines and also respond similarly when toxicity is measured by various viability criteria (Babich and Borenfreund, 1991). The two assays more frequently used for the assessment of general cytotoxicity are tetrazolium salt reduction (MTT) and neutral red (NR) assays (Castaño and Gómez-Lechón, 2005; Fent, 2001). The MTT assay is based on the measurement of mitochondrial metabolic activity by detection of the reduction of the soluble yellow MTT tetrazolium salt to purple MTT formazan by action of mitochondrial succinate-dehydrogenase (Mosmann, 1983). The NR assay is based on the accumulation of neutral red dye in lysosomes of viable cells (Borenfreund and Puerner, 1985). Other less frequently used assays are release of lactate dehydrogenase, crystal violet (CV) staining, decrease in ATP levels and cell proliferation assays. Usually these tests are performed on cultures after 72 h exposure to toxicant and are considered as basal citotoxicity tests. Cytotoxicity using fish cells has been assessed for a variety of different environmental contaminants including heavy metals, (Tan et al., 2008), water samples (Dayeh et al., 2002), industrial effluents (Davoren et al, 2005), pharmaceutical compounds (Caminada et al., 2006), endocrine disrupting compounds (Radošević et al., 2011) and others. However, before being accepted as an alternative to

in vivo fish bioassays, *in vitro* fish cell tests should be able to generate comparable results on relative potency ranking and effect concentrations of toxicants, and they should give no false positive or false negative results (Segner, 2004). It has been found that *in vitro* fish cell cytotoxicity is positively correlated with acute toxicity *in vivo*. Cytotoxicity was found in several studies to correlate with *in vivo* acute fish toxicity in different cell lines which indicates that fish cells may be useful for the prediction of acute fish toxicity for tested toxicants (Castano and Gómez-Lechón, 2005).

Cell culture assays also have been applied in a comparative approach to fish in order to assess the toxicity of different kinds of water samples. These studies have mainly originated from Canada and Germany. In Canada, effluents from pulp and paper mills, mining operations and metal processing plants were tested using RTG-2 cell line as well as RTgill-W1 and FHM cells. Usually, cytotoxicity was evaluated 24-48 h exposures by different combination of endpoints (Bols et al., 2005). In Germany, the test to which cell culture data were compared in an attempt to replace fish tests originated from a 48 h acute fish test using the golden ide. In this test, the dilution of waste water that was not lethal to fish was determined (German Industrial Standard DIN 38412-L31). Thus, the benchmark for *in vitro/in vivo* comparison in Germany was more quantitative than in Canada and in fact, a round-robin test with the RTG-2 cell line assay failed to be implemented in Germany as an alternative because its sensitivity was found to be too low (Segner, 2004).

To conclude, aquaculture has become the fastest growing food-producing sector in the world during the last three decades. It is an increasingly important contributor to national economic development, the global food supply and food security but also caused environmental and fish health problems in cases of transgenic fish. Thus, the international regulatory framework for aquatic biotechnology and an adequate mechanism to facilitate technology transfer and technical assistance must be designed in order to ensure the safe and equitable use of this technology. In aquatic toxicology fish cell culture systems represent a promising tool for basic and applied research for routine assays and screening protocols and it can be suggested that for initial priority ranking of chemicals based on *in vitro* basal cytotoxicity tests, fish cells can be used for replacing acute fish bioassays.

Literature

1. Ackermann, G., Fent, K. (1998) The adaptation of the permanent fish cell lines PLHC-1 and RTG-2 to FCS-free media results in similar growth rates compared to FCS-containing conditions. Mar. Environ. Res. 46, 363 – 367.
2. Aerni, P. (2001) Aquatic Resources and Technology: Evolutionary, Environmental, Legal and Developmental Aspects. Science, Technology and Innovation Discussion Paper No. 13. Cambridge, MA, USA: Center for International Development.

3. Babich, H., Borenfreund, E. (1991) Cytotoxicity and genotoxicity assays with cultured fish cells: a review. Toxicol in Vitro 5:91-100

4. Bols, N., Dayeh, V.R., Lee, L.E.J., Schirmer, K., 2005. Use of fish cell lines in the toxicology and ecotoxicologyof fish. Piscine cell lines in environmental toxicology. In: Mommsen, T.P., Moon, T.W. (Eds.), Biochemistry and Molecular Biology of Fishes, vol. 6, Environmental Toxicology, Elsevier B.V., pp. 43-84.

5. Borenfreund, E., Purner, J.A. (1985) Toxicity determined in vitro by morphological alterations and neutral red absorption. Toxicol Lett 24:119-124

6. Caminada, D., Escher, C., Fent, K. 2006. Cytotoxicity of pharmaceuticals found in aquatic systems: Comparison of PLHC-1 and RTG-2 fish cell lines. Aquat. Toxicol. 79, 114-123

7. Castaño, A., Gómez-Lechón, M.J. 2005. Comparison of basal cytotoxicity data between mammalian and fish cell lines: a literature survey. Toxicol. In Vitro 19, 695-705.

8. Castaño, A., Bols, N., Braunbeck, T., Dierickx, P., Halder, M., Isomaa, B., Kawahara, Lee, L.K.E., Mothersill, C., Pärt, P., Repetto, G., Riego Sintes, J., Rufli, H., Smith, R., Wood CóSegner, H. 2003. The Use of Fish Cells in Ecotoxicology: The Report and Recommendations of ECVAM Workshop 47. ATLA 31, 317-351.

9. Cvjetko, M., Radošević, K., Tomica, A., Slivac, I.. Vorkapić-Furač, J., Gaurina Srček, V. 2012. Cytotoxic effects of imidazolium ionic liquids on fish and human cell Lines. Arh. Hig. Rada Toksikol. 63, 15-19

10. Davoren, M., Ní Shúilleabháin, S., Hartl, M.G., Sheehan, D., O'Brien, N.M., O'Halloran, J., Van Pelt, F.N., Mothersill, C. 2005. Assesing the potential of fish cell lines as tools for the cytotoxicity testing of estuarine sediment aqueus elutriates. Toxicol. In Vitro. 19, 421-431.

11. Dayeh, V.R., Schirmer, K., Bols, N.C. (2002) Applying whole-water samples directly to fish cell cultures in order to evaluate the toxicity of industrial effluent. Water Res. 36, 3727-3738.

12. Fent, K., 2001. Fish cell lines as versatile tools in ecotoxicology: assessment of cytotoxicity, cytohrome P4501 A induction potential and estrogenic activity of chemicals and environmental samples. Toxicol. in Vitro 12, 477-488.

13. Gaurina Srček, V., Radošević, K., Dukić, B. 2013. Adaptation of permanent fish cell line CCO for growth in serum-free media. Curr. Opi. Biotech. 24, Supplement 1, S63-S63.

14. Kniewald, J., Kmetič, I., Gaurina Srček, V., Kniewald, Z., 2005. Alternative models for toxicity testing of xenobiotics. Arh. Hig. Rada Toksikol. 56, 195-204.

15. Kniewald, J., Kniewald Z., 2007. The Ocean and its Environment as a Source of Food Production, Ann. Croat. Acad. Eng. 2006. pp. 15-36, ISSN 1332-3482.

16. Lakra, W.S., Swaminathan, T.R., Joy, K.P. (2010). Development, characterization, conservation and storage of fish cell lines: a review. Fish Physiol. Biochem. 37, 1-20.

17. Mosmann, T. (1983) Rapid colorimetric assay for cellular growth and survival, application to proliferation and cytotoxic assays. J. Immunol. Methods 65:55-63

18. Radošević, K., Tonković, T., Slivac, I., Kniewald, Z., Gaurina Srček, V. 2011. Comparison of cytotoxicity induced by 17α-ethynylestradiol and diethylstilbestrol in fish CCO and mammalian CHO-K1 cell lines. Bull. Environ. Contam. Toxicol. 86, 252-257.

19. Rocha, A., Ruiz, S., Estepa, A., Coll, J.M. 2003 Fish as biofactories: inducible genetic systems and gene targeting. Span. J. Agri. Res. 1, 3-11.

20. Segner, H. (2004) Cytotoxicity assay with fish cells as an alternative to the acute lethality assay to fish. ATLA 32, 375-382.

21. Slivac, I., Gaurina Srček, V., Radošević, K., Porobić, I., Bilić, K., Fumić, K., Kniewald, Z. 2008. Growth characteristics of Channel Catfish Ovary cells – influence of glucose and glutamine. Cytotechnology 57, 273-278.

22. Slivac, I.,Blajić, V., Radošević, K., Kniewald, Z., Gaurina Srček, V. 2010. Influence of different ammonium, lactate and glutamine concentrations on CCO cell growth. Cytotechnology 62, 585-594.

23. Sixth Report on the Statistics on the Number of Animals used for Experimental and other Scientific Purposes in the Member States of the European Union, 2010) COM (2010) 511

24. Tan, F., Wang, M., Wang, W., Lu, Y. (2008) Comparative evaluation of the cytotoxicity sensitivity of six fish cell lines to four heavy metals in vitro. Toxicol. in Vitro 22:164-170

25. Wolf, K., Quimbly, M.C. (1962) Established eurythermic line of fish cells in vitro. Science 135, 1065-1066.

Aflatoxins Fifty Years Later: Still Unsolved Challenge

Slaven Zjalić* *Review*

Department of ecology, agronomy and acquaculture, University of Zadar,
Trg kneza Viseslava 9, 23000 Zadar

Aflatoxins were discovered after the massive death of turkeys in the UK more than 50 years ago. These toxins, secondary metabolites of moulds classified in *Aspergillus* section Flavi, present a high health hazard due to their cytotoxic, genotoxic immunosuppressive and teratogenic effects on humans and animals. Therefore, the concentration of aflatoxins in food and feed commodities is limited by law in almost all countries. The contamination with aflatoxins can occur on different plant and animal derived substrates (seeds, dried fruit, cheese, dried meat), but seeds, especially the oily ones, are considered the major vehicle of aflatoxins in the food chain. After the ingestion, aflatoxins can be transmitted along the food chain either unchanged or metabolised in other more or less toxic forms, like aflatoxins M_1 in milk. The aflatoxigenic moulds require relatively low water activity for their growth ($a_w \geq 0.7$) and consequently contamination of seeds with aflatoxins can occur in the field or during any phase of postharvest processing and storage. In the last decade, probably due to climate changes, the contamination in the fields also occurs more frequently in European countries where, in the past, the presence of aflatoxins in food and feed was related to imported raw materials or inadequate storage. Different strategies, either prevention or detoxification, have been applied to control the presence of aflatoxins in food and feed but none of them has completely solved the problem. The research of new, more environmentally friendly strategies and tools in aflatoxin control is still ongoing.

Key words:
aflatoxins, toxicity, maize, control, food safety,

Introduction

Mycotoxins, secondary metabolites of some filamentous fungi that are toxic for humans and animals, are common natural contaminants of feed and foodstuff. These molecules have no particular odor, taste or color by which their presence in food or feed could be detected. Therefore, throughout the history they were present in diet and posed a serious health risk. After the discovery of the first mycotoxins

*Corresponding author: szjalic@unizd.hr

in early 1960's, several authors have illustrated and hypothesized their possible influence on socio-politic events and human history, from plague epidemics and the witches of Salem to the short life expectancy of medieval man (Caporel, 1976; Matossian 1984, 1986, 1989; Woolf, 2000). More recently, Bennett and Klich (2003) have reviewed the possibility of using mycotoxins as biological weapon. Presently, nearly 400 fungal secondary metabolites have been classified as mycotoxins, but only about a dozen of them pose serious health hazard for humans and animals and are therefore the object of intensive scientific research and legislation limits (Keller et al., 2005). Aflatoxins, the first mycotoxins discovered, are among the most dangerous ones for human and animal health, and among the most studied mycotoxins. However, more than 50 years after their discovery, the method for complete control of their presence in food and feed has not been achieved. In this paper the actual knowledge on aflatoxins, is reviewed.

Aflatoxins and aflatoxigenic fungi

Already at the end of the 19th century scientists suggested that some illnesses could be related to the consumption of food and feed contaminated by moulds. In 1957, Burnside and collaborators published a scientific paper describing pathologies in pigs fed with maize contaminated by *Aspergillus*, but no causing agent was identified (Burnside et al., 1957; Christensen, 1975). A few years later, in 1960, an outbreak of unknown illness, turkey X disease, killed more than 100,000 turkeys and few thousands of other poultry on different farms around the UK. The only thing in common was the component of feed based on peanut meal from Brazil. Among various hypotheses on the causes of death of the poultry, the contamination by moulds was taken into consideration and it soon became clear that the disease was caused by metabolites of *Aspergillus flavus.* These metabolites were named, after the mould that produced them, aflatoxins (Sargent et al., 1963).

Aflatoxigenic fungi can produce four different aflatoxins, named after their blue (B) or green (G) fluorescence under the UV light (λ = 360 – 365 nm) and their relative mobility by thin-layer chromatography on silica gel (B1 and B2, G1 and G2). Some strains and genera produce only B group aflatoxins (i.e. *A. flavus*) while the others (i.e. *A. parasiticus*) produce both groups (Klich and Pitt, 1988).

Today, there are about 20 different aflatoxins. Four of them are produced by aflatoxigenic fungi, while the others are hydroxylated derivates of fungal toxins produced by animal metabolism during the detoxification process. Probably the most known is aflatoxin M1, which is typically produced by mammalian metabolism from aflatoxin B1 and secreted in the milk (Pitt and Hocking, 1985; Keller et al., 2005). Generally,

aflatoxins derived from animal metabolism are less toxic than the original ones. For example, aflatoxin B1, which is the most toxic one for animals and humans, is among the most powerful natural occurring mutagens (Eaton and Groopman, 1998; Bennet and Klich 2003) classified by IARC (International Agency for Research on Cancer) in group 1 – carcinogenic substances, while its animal metabolic derivate M1 is classified in group 2b – potential carcinogenic substances (IARC, 2013).

Aflatoxins are stable and heat tolerant molecules (Pit and Hockin, 1985). They are quite heat-resistant up to their melting point, which is between 240 and 299 °C. In a solution the degradation can occur on lower temperatures after longer exposition. In oily seeds boiled in water or roasted it was observed after 60 to 120 minutes at temperatures around 120 °C – 150 °C (Adegoke et al., 1994; Yazdanpanah et al., 2005). In any case, aflatoxins are resistant enough to persist any heat treatment normally used in food and feed preparation.

The production of aflatoxins has been reported for different species of genera *Aspergillus* (*Aspergillus flavus*, *A. parasiticus*, *A. nomius*, *A. pseudotamarii*, *A. bombycis*, *A. toxicarius*, *A. parvisclerotigenus*, *A. minisclerotigenus*, *A. arachidicola*, *A. pseudonomius*, *A. pseudocaelatus*) and some ascomycetes like *Emericella spp.* and *Pteromyces spp.* (Van Waalbeek et al. 1968; Kurtzman et al, 1987; Sun and Qi, 1991; Saito et al 1993; El-kady et al., 1994; Frisvad et al, 1999; Klich et al, 2000; Ito et al, 2001; Peterson et al., 2001; Frisvad and Samson, 2004; Frisvad et al., 2004; Cary et al., 2005; Frisvad et al 2005). Some new aflatoxigenic species were isolated in Portugal and described recently: *Aspergillus mottae*, *A. sergii* and *A. transmontanensis* (Soares et al., 2012), but still a little is known about their distribution and incidence. The major part of contaminations with aflatoxins in agriculture fields is caused by *A. flavvus* and *A. parasiticus* (Chang et al., 2007). Some of the other fungi reported as aflatoxin producers have limited habitats and distribution, generally in the tropics, while the others can synthesize aflatoxins only under certain conditions rarely present in nature, and therefore their contribution to aflatoxin contamination in agriculture fields is negligible compared to *A. flavus* and *A. parasiticus* (Cary et al., 2005). Some studies indicate that the role of some species, like *A. nomius,* in aflatoxin contamination could be underestimated, and in future their importance in aflatoxin contamination in agricultural crops in some geographical areas could be revised (Varga et al., 2011).

Aspergillus is a genus of mitosporic fungi (also known as fungi imperfecti or Deuteromycota) that include about 100 species. Both *A. flavus* and *A. parasiticus,* like many other aflatoxigenic *Aspergillus,* belong to *Aspergillus* section Flavi. Not all the fungi belonging to section Flavi are producers of aflatoxins. Two species closely related to *A. flavus and A. parsiticus*, *A. oryzae* and *A. sojae* respectively, used in food industry are not toxin producers and are considered as GRAS (Chang et al., 2007). *A. flavus* and *A. parasiticus* are saprophytic fungi that occur in soil and are

widespread all over the world surviving on many organic nutrient sources, like plant debris, animal fodder or dead insects (Pitt and Hocking, 1985; Amaike and Keller, 2011). In certain conditions both *A. flavus* and *A. parasiticus* can act as weak plant pathogens affecting different important agricultural crops such as maize, peanuts or cottonseeds. They can grow at temperatures ranging from 12 to 48 °C with optimum between 28 and 37°C (Yu, 2012). For most of its life cycle, under favorable conditions, fungus grows in the form of mycelium or asexual spores, conidia, and under adverse conditions develops resistant structure, sclerotia. Sclerotia can survive in the soil under severe environmental conditions and then produce conidia under favorable conditions (Wicklow et al., 1993). Conidia can be spread either by wind or water. In infections of aboveground crop seeds, like maize, dispersion of conidia by wind plays an important role while the dispersion by water is more important for infections of peanuts and cottonseeds (Horn et al. 2001; Amaike and Keller 2011.). Insects and birds can enhance the incidence of infections by damaging kernels which in turn provides entry sites for fungal penetration (Ni et al., 2011). Moreover, insects can be vectors of aflatoxigenic fungi, which seem particularly important during postharvest infections during storage (Nesci et al., 2011).

Generally, fungi belonging to genus *Aspergillus* need relatively low water activity ($a_w \geq 0.60 – 0.70$) for their growth, and aflatoxigenic strains are not an exception, and therefore they can contaminate crops in the field and during the storage (Pitt and Hocking, 1985; Klich and Pitt, 1988).

The two major aflatoxin producers, *A. flavus* and *A. parasiticus* have different host specificity. More precisely, *A. flavus* seems to have no host specificity and affects a wide range of hosts, contaminating the seeds produced above the ground, while *A. parsiticus* contaminates seeds under the ground, like peanuts (Amaike and Keller 2011, Yu, 2012). *A. parasiticus* seems to be dominant in sugar cane yards. This difference is more likely connected to the adaptation of isolates to specialized niches in which they have competitive advantage than to the host specificity. Furthermore, all strains of *A. parasiticus* produce all four aflatoxins – B1, B2 and G1 and G2 – while the major parts of the strains of A. flavus produce only B aflatoxins. *A. flavus* strains can have large (L) or small (S) sclerotia, strains L produce only B1 and B2 aflatoxins, but strains S produce all four (Abbas et al. 2005).

Toxicology

All four main aflatoxins are toxic for humans and animals with mutagenic, teratogenic and immunosuppressive effects (Goldman and Shilds, 2003; Wangikar et al.,

2005; Tessari et al., 2006; Yijang et al., 2006). Aflatoxins can have both cytotoxic and genotoxic effects on humans and animals. All four aflatoxins produced by fungi are mutagenic – B1 is the most mutagenic, followed by B2, G1 and G2 (El-Zawahri et al., 1990; Goldman and Shilds, 2003). Other aflatoxins are formed in animals and humans as the result of detoxification by hydroxylation of the aflatoxins present in feed and food after the ingestion of contaminated meal. Members of cytochrome P 450 monoxygenases family are involved in detoxification and hydroxylation (Karenlampi, 1987). These isoenzymes produce different products, some of which have a low toxicity, like aflatoxin P1, while the others are more toxic than aflatoxins from which they derive (Angsubharkon, 2000). The enzymes of cytochrome P 450 family are differently expressed in various tissues of the same organism and in different species, resulting in diverse animal and tissue susceptibility to aflatoxins (Eaton and Gallhager, 1994). Diverse cytochrome P 450 isoenzymes are present in liver, including P450 3A5, which catalyzes transformation of aflatoxin B1 into more toxic and cancerogenic by-product, 8,9-epoxide of aflatoxin B1. This molecule binds to the guanine residues in DNA, forming guanil- N7 adducts, and induces mutation. One mutation hot spot is the transition of G to T at the third base of codon 249 of the p53 onco-suppressor gene. This mutation compromises the function of the protein p53, a regulator of cell cycle, and this is generally believed to be the mechanism for initiating hepatocarcinoma insurgence (Smela et al., 2001; Lereau et al., 2012). Another cytochrome, P 450 monoxygenase, in liver converts aflatoxin B1 into aflatoxin B2a, a highly cytotoxic molecule. This aflatoxin interferes with lipid and carbohydrate metabolism in liver by blocking certain enzymes of these metabolic pathways. The result is the accumulation of lipid in hepatocyte cytoplasm and necrosis. Due to hepatic dysfunctions, different metabolic pathways in organism are affected, resulting in diffuse internal bleeding and, in the end, the death of the animal. Aflatoxin B2a is probably largely involved in insurgence of symptoms of acute aflatoxicosis (Angsubhakorn, 2000). In mammalian glands another P 450 monoxygenase catalyzes conversion of aflatoxin B1 into aflatoxin M1, which is secreted in the milk. Aflatoxin M1 is less genotoxic than B1, as previously explained, but is cytotoxic without the need of metabolic activation (Neal et al., 1998, Caloni et al., 2006).

Aflatoxins are toxic for all vertebrates even if the lethal dose (LD 50, dose at which 50% of tested population dies) varies among different species and, sometimes, even among the animals of different sex of the same species. For example in rabbits LD50 is 0.30 mg kg $^{-1}$ body, in rat male 7.20 mg kg $^{-1}$ body weight and in rat female 17.90 mg kg $^{-1}$ body weight (Caradona et al., 2000). The susceptibility to aflatoxins also depends on the age, nutritional status of the animal, as well as on the animal's general health conditions.

Acute aflatoxicosis in animals has occurred many times, and turkey X disease is one of the examples. The outbreak of aflatoxicosis has occurred a few times

in the last 20 years in domestic animals (dogs) fed by the feed contaminated with aflatoxins (Garland and Reagor, 2001; Dereszynski et al, 2008). Different cases of human acute aflatoxicosis were also reported: in Taiwan in 1967 (Ling et al., 1967), Uganda in early 1970's (Serch-Hanssen, 1970), Kenya (Pitt, 2000), Thailand (Shank et al., 1971), Czechoslovakia (Dvorachova et al., 1972), United States (Chaves-Carballo et al., 1976) and Germany (Rosinberg, 1972). The last one happened in Kenya in 2004 when 125 out of 317 intoxicated people died. In this case, all the intoxicated persons were from three neighboring villages who consumed poorly stored maize of their own production (Azziz-Baumgartner et al., 2005). Generally, acute aflatoxicosis is caused by contamination during the storage, not in the field (Amaike and Keller, 2005). Due to their higher metabolic activities, children are more severely affected by aflatoxicosis. Moreover, aflatoxicosis is considered to be the cause of several human diseases that particularly affect children, like Ryes syndrome or Kwashiorkor (Angsurbharkon, 2000).

The continuous consumption of lower quantities of aflatoxins can present health hazard, too. In different animals (piglets, turkeys, chicken, hamsters, rabbits) the immune depression caused by the consumption of feed contaminated with aflatoxins has been observed (Hegazy et al., 1991; Azzam and Gabol., 1998; Fink Gremmels 1999; Whirlow and Hagler, 2002; Shivanchandra et al., 2003: Tessari et al., 2006). Furthermore, aflatoxins in sublethal concentration for animals have teratogenic effects. Malformations of the skeleton, blood vessels, locomotory system, kidneys and death of a fetus have been reported (Roll et al., 1990; Wangikar et al., 2005). Therefore, the ingestion of sublethal quantities of aflatoxins can cause severe economic losses to cattle, piglet and poultry breeders due to major susceptibility to illnesses, impaired growth, weight losses and insurgences of anorexia (Proctor, 1994). The presence of aflatoxins can impair the growth of children. This problem is particularly present in the developing countries (Yijanget al., 2006; Khlangwiset et al., 2011; Smith et al., 2012).

Another important issue is the transmission of aflatoxins along the food chain. Traces of aflatoxins, B1 or its derivates, have been found in different animal organs and tissues (Angsubharkon, 2000). In this way, aflatoxins ingested by an animal can reach the final consumer – man. Fortunately, only a minimum part of the ingested aflatoxins is found in animal tissues. Table 1 shows the ratios of ingested aflatoxins and aflatoxins found in some animal products. It is important to specify that the toxicity of aflatoxins and their transmission depend on numerous factors, including the age of the animal, its health conditions, type of nutrient, and that the reported data are just indicative (Whitlow and Hagler, 2002; Vallone et al., 2006).

Table 1 – Aflatoxins detected in animal tissues of animals fed with aflatoxin B1 contaminated diet. Data are expressed as ratio of the quantity of toxins present in diet (ppm) and the quantity of toxin detected in tissues (from Angsubharkon, 2000).

Animal	Tissue	Aflatoxin	Ratio feed/tissue
Poultry	Eggs	B1	2,200
Ruminants	Milk	M1	300
Ruminants	Liver	B1	14,000
Swine	Liver	B1	800
Broiler	Liver	M1	1,200

Legislation

To minimize the potential exposure of population to aflatoxins legislators of almost all countries in the world have set the legal limits for the presence of aflatoxins in food, feed and their raw materials. The limits are not homogenous among the countries. In EU regulations the limits are rather strict. For example, the allowed quantity of aflatoxins in cereals is 5 ppb (5 mg kg^{-1}), while in the USA and many Asian and African countries the limit is 20 ppb. The same applies to aflatoxin M1 – in EU countries the limit is 0.05 ppb, while in the USA and a number of other countries this limit is 10 times higher (Price et al., 1993; Bhatnagar et al., 2004; Lewis et al., 2005). In all legislations the limits can vary according to the destination of the product. The most rigorous limits are for children food (i.e. aflatoxin M1 in milk) and less restrictive for feed production. The quantity of average intake of food or feed commodity is also considered by legislators. The presence of higher quantity of aflatoxins in cayenne pepper does not represent the same health risk for consumers as the presence of higher quantity of toxins in maize or milk. Therefore, the limits for spices are higher than those for other foodstuff more common in human diet. Aflatoxins B1 and M1 are particularly discussed and their presence and quantity are defined by the law. The former is the most toxic one and largely produced by fungi, while the latter is present in milk and its derivates, and in children food. The other aflatoxins, B2, G1 and G2, are produced by fungi in lower quantities and only when aflatoxins B1 is produced, and are thus considered only as a sum of total aflatoxins. The presence of aflatoxins in food and feed in the EU countries is regulated according the Regulation 1881/2006 and successive amendments – 1126/2007, 565/2008, 629/2008, 105/2010 and 165/2010. The complete regulation can be found at the EU web pages, ec.europa.eu./food/food/chemicalsafety/contaminants/aflatoxins_en.htm.

Genetics and molecular biology of aflatoxin biosynthesis

The extensive research that followed aflatoxin discovery enabled us to understand the synthetic pathway of these toxins and the genes responsible for their biosynthesis. The milestone was the individuation of a mutant accumulating brick-red pigment, norsolinic acid (NOR), the first stable aflatoxin precursor (Bennett et al., 1983). This discovery led to the identification of other intermediates of aflatoxin biosynthetic pathway and, in the end, individuation of genes, enzymes and regulators involved in aflatoxin synthesis.

The first step in this pathway neither is the synthesis of NOR catalyzed by two types of enzymes, FAS and PKS. FAS are a fatty acid synthase different from those normally used in lipid metabolism, and two different FAS enzymes are involved in aflatoxin synthesis, FASα and FASβ (Brown et al., 1996). PKS is a polyketide synthase, large enzyme containing four domains typical for all known PKS proteins – β-ketoacyl synthase, acetyltransferase, acyl carrier protein and thioesterase (Crawford et al., 2008a). The initial substrate is a hexanoil starter unit which is converted to norsolinic acid anthrone, noranthrone (Crawford et al., 2006; Crawford et al., 2008b). The conversion of noranthrone to norsolinic acid is not completely clear – it could be due to the action of some oxidase or monoxygense, but it could also occur spontaneously (Dutton, 1988). The following stepsin biosynthetic pathway are: conversion of NOR to averantin (AVN) catalyzed by a ketoreductase, conversion of AVN to 5-hydroxyaverantin HAVN catalyzed by a monooxygenase, conversion of HAVN to averufin (AVN) via 5'-oxaverantin (OAVN) catalyzed by an alcohol dehydrogenase, conversion of AVN to versiconal hemiacetal acetate (VHA) catalyzed by a monooxygenase, conversion of VHA to versiconal (VHOH or VAL) catalyzed by an esterase, conversion of VHOH to versicolorin B (VER B) catalyzed by a cyclase (Yu, 2012). VER B is a branch point for synthesis of aflatoxins B1 and G1 or B2 and G2. It can be converted to versicolorin A (VER A) by a monooxygenase leading to the synthesis of aflatoxins B1 and G1or it can be transformed directly by the same enzymes that catalyze the conversion of VER A toward the synthesis of B2 and G2. The enzymes involved in further steps of the synthesis of aflatoxins B1 and G1 and aflatoxins B2 and G2 are the same, but the substrate are different thus the products are different too. It seems that cultural conditions could have an important role in the conversion of VER B to VER A thus affecting the final ratio of aflatoxins B1 to B2 and G1 to G2 (Yabe and Nikajima, 2004). The aflatoxin synthesis continues as follows: conversion of VER A to demethylsterigmatocystin (DMST) or conversion of VER B to demethyldihydrosterigmatocystin (DMDHST) catalyzed by an enzyme complex consisting of four enzymes, one ketoreductase, two monooxygenases and an enzyme, OrdB, whose role is still unclear; conversion of DMST to sterigmatocystin (ST) or conversion of DHDMST to dihydrosterigmatocystin (DHST) catalyzed by two O-methyltransferases; conversion of ST to O-methylsterigmatocystin or conversion of DHST to dihydro-O-methylsterigmatocystin (DHOMST) catalyzed by an O-methyltransferase. The

final conversion of OMST to aflatoxins B1 and G1 or conversion of DHOMST to aflatoxins B2 and G2 involves different enzymes. Synthesis of B-group aflatoxins involves a monoxygenaseOrdA, while the synthesis of G-group involves two enzymes: NadA and NorA. The genes of these two enzymes are incomplete in fungi that do not produce G aflatoxins (Yu, 2012).

The genes of proteins involved in aflatoxin synthesis are clustered, as it is often the case with the genes of secondary metabolism (Yu and Keller, 2005). As many as 30 genes are potentially involved in aflatoxin synthesis (Yu, 2012). The cluster occupies a 75 kb region of fungal genome near one telomere of chromosome III (Georgianna and Payne, 2009). The genes involved in aflatoxin synthesis were discovered by different research groups in the time span of nearly 15 years and some of them were first cloned in *A. nidulas*, the producer of sterigmatocystin, and therefore they were first named according to their catalytic role. In 2004 Yu and collaborators proposed a new nomenclature for genes of aflatoxin cluster – all genes are named *afl* followed by a letter. This nomenclature is now in use; nevertheless the good practice should be to put the new name followed by the old denomination in brackets. The aflatoxin pathway genes are expressed concurrently. Two genes regulate aflatoxin gene expression, *aflR* and *aflS* (*aflJ*). The first one, located in the middle of the pathway, encodes a positive-acting transcriptional factor AflR, a 47 kDa sequence specific DNA-binding protein ($Zn(II)_2Cys_6$) required for transcriptional activation of genes of the aflatoxin gene cluster. It binds the consensus motif 5'TCGN$_5$CGR3' located in promoter region of many aflatoxin genes (Woloshuk et al., 1994; Chang et al., 1995, 1999; Ehrlich et al., 1999; Yu et al, 1996, 2004; Yu, 2012). The overexpression of this gene up-regulates the expression of genes in aflatoxin biosynthetic pathway and increases aflatoxin production. The fungi with deletions or mutations of *aflR* are unable to produce aflatoxins (Flaherty and Payne, 1997; Price et al., 2006). Even if AflR is essential for aflatoxin synthesis its presence alone is not sufficient and another regulator, *aflS*(*aflJ*), is required. This gene is situated adjacent to *aflR* and encodes a protein that shows no DNA-binding properties. Overexpression of this gene has no influence on aflatoxin biosynthesis (Du et al., 2007). Nevertheless, the deletions of this gene result in inhibition of aflatoxin synthesis (Meyers et al., 1998, Chang, 2004; Du et al., 2007). It is considered that AflS interacts with AflR, maybe as an activator. Recent researches indicate that AflS could be involved in the transport of AflR to or out of the nucleus, controlling the availability of AflR for transcription activation (Echrlih et al., 2012).

The aflatoxin cluster is present, more or less conserved, in different fungi non-producers of aflatoxins, both in *Aspergillus spp.* and in some distantly related genera (Anaike and Keller, 2012). Due to the mutations and deletions in these fungi, cluster is not functional. In the genome of two *Aspergillus* species normally used in food industry, *A. oryzae* and *A. sojae*, the aflatoxin gene cluster is present but it contains mutations and deletions, whicg disable aflatoxin biosynthesis. These mutations/deletions often affect a*flR* and *aflS(aflJ)* expression (Price et al., 2004; Chang, 2004; Chang et al., 2007).

Another two proteins not codified by aflatoxin gene cluster are involved in the regulation of aflatoxin synthesis, LaeA, VeA and VelB. Genes of these proteins, first found in *A. nidulans,* are well conserved in all fungi. The global regulatory gene *laeA* (lack of *aflR* expression) is a nuclear protein that activates some genes not associated with secondary metabolism and several secondary metabolism gene clusters, like aflatoxin cluster in *A. flavus* and *A. parasiticus,* sterigmocystin and penicillin clusters in *A. nidulans* or virulence genes in *A. fumigatus* (Bok and Keller, 2004; Perrin et al., 2007; Bouhired et al., 2007; Kale et al., 2007, 2008). The position of a gene in genome is important for its regulation by LaeA. When *aflR* was placed in a genome out of the aflatoxin gene cluster, its regulation was not affected by LaeA (Bok et al., 2006). In *A. nidulans* VeA is crucial in light-dependent conidiation (Mooney and Yager, 1990; Bayrum et al., 2008). In light conditions *A. nidulans* forms conidia, whereas in dark conditions it preferentially develops cleistothecia. During the sexual development, the expression of *veA* gene increases. In *A. nidulas* it was observed that light impairs the transport of VeA into the nucleus, therefore in light conditions it is located mainly in cytoplasm. In dark conditions VeA migrates into the nucleus where it regulates light-dependent development and sterigmatocystin (ST) synthesis (Fisher, 2008). In *A. flavus* and *A. parsiticus* deletion of *veA* resulted in impaired aflatoxin production and sclerotia formation, while overexpression of *veA* shows major production of sclerotia and higher aflatoxin production. Similar results were observed in strains with deleted or overexpressed *leaA* (Duran et al., 2007; Amaike and Keller, 2009). Both LaeA and VeA are essential for full virulence in different fungi, including *A. parasiticus* and *A. flavus* (Amaike and Keller, 2009). VelB is another protein conserved in fungal kingdom, and it shares 18% of amino acid identity with VeA, but has no typical nuclear localization signal. VeA forms a complex and enhances the transport of VelB into the nucleus. LaeA, VeA and VelB associate in heterotrimeric nuclear complex, Velvet complex, which is well conserved in filamentous fungi (Bayrum et al., 2008). It is hypothesized that LeaA, and probably all the Velevet complex, is involved in histone modification that regulates the accessibility of genes for transcription by acetylation of histone H4 (Roze et al., 2007a, b; Reyes-Dominguez, 2010). Histone modifications are involved in regulating the gene expression. The pattern of histone posttranslational modifications (PTMs) might affect the structure of chromatin or modify distinct binding regions of transcriptional factors (Fischle et al., 2005; Hirota et al., 2005).

Factors affecting aflatoxin biosynthesis

A range of factors influence aflatoxin biosynthesis: nitrogen and carbon sources, temperature, water activity, pH, and bioactive genes. The best known nutritional factors affecting aflatoxin biosynthesis are carbon and nitrogen sources (Reverberi

et al., 2010). The influence of carbon source on aflatoxin synthesis has been widely studied. Regarding carbohydrates, aflatoxin production is supported by simple sugars like glucose, sucrose, fructose and maltose, but not by sorbose or lactose (Payne and Brown, 1998). It seems that the key factor that determines whether a carbon source can support aflatoxin biosynthesis is its availability to both hexose monophosphate and glycolytic pathways (Price et al., 2006). The long chain saturated lipids are another source of carbon that supports *A. flavus* growth but not aflatoxin biosynthesis. On the contrary unsaturated fatty acids support aflatoxin biosynthesis but affect fungal growth (Fabbri, 1983; Fanelli 1985).

Different amino acids and ammonium compounds, like proline, asparagine, alanine, ammonium nitrate, and ammonium sulfate support aflatoxin production (Reddy et al., 1979; Payne and Hagler, 1983; Payne and Brown, 1998). Interestingly, the presence of tryptophane culture media is reported to significantly enhance the synthesis of aflatoxin production in *A. parasiticus*, but impairs the biosynthesis of aflatoxin B1 in *A. flavus*. Tryptophane acts as up-regulator in *A. parasiticus* and as down-regulator in *A. flavus* (Wilkinson et al 2007.). Sodium nitrate and sodium nitrite containing media are not conducive for aflatoxin production (Reddy et al., 1979). Nitrate has been reported to enhance AflR synthesis and transcription, but also to have suppressive effects on aflatoxin synthesis. This apparent contradiction could be attributed to the affinity of AreA, nitrogen regulatory gene in *A. parasiticus* for binding AflR and AflS (AflJ) and consequent inhibition of dimerization between AflR and AflS (AflJ); in fact several binding motifs for AreA on AflR and AflS were identified (Chang et al., 2000).

Aflatoxin biosynthesis is optimal at the temperature between 28 and 30 °C, and above 37 °C it is completely inhibited. O'Brian and collaborators (2007) observed a remarkable decrease in the expression of aflatoxin genes in cultures incubated at 37 °C in comparison with those incubated at 28 °C, but this decrease was not so evident for *aflR* and *aflS*(*aflJ*). More recently, it was demonstrated that the expression of both genes is under-regulated by high temperature. The under-regulation affects more *aflS*(*aflJ*) than *aflR*, and the change in the ratio of two proteins probably causes non-functionality of AflR in transcription activation.

Another factor that influences aflatoxin biosynthesis is pH. Production of aflatoxins in *A. flavus* occurs in acidic media, while it is inhibited in alkaline ones (Cotty, 1988). A binding site for PacC, a transcriptional regulatory factor, has been identified in the *aflR* promoter region. PacC, studied in multiple fungal species, is an activator of genes expressed in alkaline media and a repressor of those expressed in acidic ones (Penalva et al, 2008; Selvig and Alspaugh, 2011). The binding of PacC on its consensus site in *aflR* promoter region could inhibit the transcription of *aflR* in alkaline solutions (Cary et al., 2006).

Oxidative stress

Oxidative stress is considered to be a prerequisite for aflatoxin biosynthesis (Jayashree and Subramanyam, 2000; Hong et al., 2013). Fungi are aerobic organisms and they need oxygen for their metabolism. Metabolic oxidation process creates, as a by-product, reactive oxygen species (ROS), which can damage cell structures (i.e. membranes). Cell produces antioxidant molecules and enzymes which maintain oxidant/antioxidant ratio in balance. In *A. flavus* and *A. parasiticus* disruption of this balance toward hyper-oxidant status induces aflatoxin biosynthesis (Reverberi at al., 2010). Accumulation of reactive oxygen species (ROS) in *A. parsiticus* cell prior to aflatoxin synthesis have been described by several authors (Jayashre and Subramanian, 2000; Zjalic, 2006; Reverberi et al., 2005, 2008). An ortholog of yeast *yap1* gene, *apyapA*, was identified in *A. parasiticus.* Yap1 is a transcriptional activator expressed in the response to oxidative stress in yeast (Toone and Jones, 1998). In its C and N-terminal regions Yap1 contains cysteine rich domains. In the presence of oxidants (H_2O_2) Yap1 is activated, two or more cysteines of C-terminal region undergo direct oxidation and an intramolecular bisulfide bound is formed. Activated Yap1 is a transcriptional factor of different genes involved in cell response to oxidative stress (Delanuay, et al., 2000; Hong et al., 2013). Disruption of *apyapA* gene in *A. parasiticus,* incubated both in synthetic liquid media and in maize seeds, resulted in major release of hydroperoxides in the media and in higher and earlier aflatoxin production, further confirming the involvement of oxidative stress in regulation of aflatoxin biosynthesis (Reverberi et al., 2005, 2007, 2008; Zjalic 2006). Already in 1980's, Fanelli's group demonstrated that oxidative stress and lipoperoxidation enhance aflatoxin biosynthesis and that some antioxidants can inhibit aflatoxin synthesis (Fabbri et al., 1983; Fanelli et al 1983, 1985). The importance of lipoperoxides for aflatoxin biosynthesis was confirmed by other researches. Different influence of two stereoisomers of linoleic acid, 9S-hydroperoxyoctadecanoic acid (9S-HPODE) and 13S-hydroperoxyoctadecanoic acid (13S-HPODE), on aflatoxin biosynthesis has been discussed. In *A. flavus* 9S-HPODE stimulates aflatoxin production, while 13S-HPODE has inhibiting effects (Burow et al., 1997). It has been established that oxylipins, a class of oxygenated unsaturated fatty acids derived from oxygenase activity (like 9S-HPODE and 13S-HPODE), are involved in signaling pathways in filamentous fungi, yeasts, oomycetes, plants and animals (Tsitsigiannis et al., 2005; Tsitsigiannis and Keller, 2007). In *A flavus* oxylipins regulate sclerotia and conidia production and secondary metabolism. They are also important in several *Aspergillus*/host interactions (Burow et al., 1997; Tsitsigiannis and Keller, 2007; Mita et al., 2007; Horowitz et al., 2009). Some authors have proposed utilization of genetically modified corn containing mainly 13S-HPODE–lipoxygenases as a tool for lowering aflatoxin contamination in maize seeds (Burow et al., 1997).

In filamentous fungi peroxisomes are important for primary metabolism (i.e. β-oxidation of fatty acids), and can play an important role in the formation of some

secondary metabolites (Lopez-Huertas et al., 2000; Maggio-Hall, 2005). Stimulation of peroxisome proliferation resulted in up-regulation of fatty acids β-oxidation, accumulation of intracellular ROS and it enhanced aflatoxin biosynthesis (Reverberi et al., 2012). Further studies are needed in order to understand better the demonstrated link between peroxisome metabolism and aflatoxin synthesis.

Presence of aflatoxins in food and feed

Two fungi that are mostly responsible for aflatoxin contamination on food and feed, *A. flavus* and *A. parasiticus*, can grow on a wide range of temperatures and substrates making the problem of aflatoxin contamination almost ubiquitous. Moreover, aflatoxigenic fungi require relatively low water activity and therefore the contamination can occur both in field and during storage. Aflatoxin contamination was detected in different food and feed stuff like cereals, dried fruit, spices, medicinal herbs, coffee, cocoa, eggs, milk, olives, and beer (Etam 1996; Pitt, 2000; Malby et al., 2005; Roussos et al., 2006; Ghitakou et al., 2006; Trucksess and Scott 2008; Rodrigues and Nsehrer 2102; Alpsoy et al, 2013; Andrade et al., 2013). Oily seeds are mostly prone to aflatoxin contamination. At the same moisture content as starchy seeds oily seeds present major water activity and therefore can be more easily exposed to colonization of aflatoxigenic fungi. Furthermore, peroxidation of their lipids stimulates aflatoxin biosynthesis hence the quantity of produced toxins is higher. For a long time, the aflatoxin contamination in Europe was rather attributed to the import and inappropriate storage conditions than to the contamination in the field. Lately, the contamination by *A. flavus* in the field was recorded in different south eastern countries and it was probably caused by global climate changes. In an Italian research, conducted from 1992 to 1998 on maize and grains of Italian production, various aflatoxin contaminations were determined. The contamination levels were not homogenous along the studied period (Haouet and Altissimi, 2003). In 2003 and 2013 climate conditions, hot and droughty summers, favored *A. flavus* contamination of maize in the fields in different European countries (Vallone et al., 2006; oral communication). The result was higher concentration of aflatoxin M1 in milk, as the recent outbreak of M1 in Croatia testifies. Nevertheless, different studies indicate that the levels of aflatoxins in feed are generally above the limit values set by the EU regulations (Piccaglia et al., 2005; Rodriguez and Naehrer, 2012; Streit et al., 2012).The Joint FAO/WHO Expert Committee on Food Additives (JEFCA) estimated that a considerable number of deaths in developing countries could be related to the consumption of food contaminated with aflatoxins (JEFCA, 2001). The situation in Europe is much better, probably due to better prevention and less favorable environmental conditions for *A. parasiticus* and *A. flavus* growth than in subtropical and tropical regions. Reports of the members of the European

Union (SCOOP, 1996) showed estimated dietary exposure for Europeans ranging from 0.03 to 1.28 ng kg^{-1}bw day^{-1}. One of the most complete researches on the presence of mycoxins in a European country has been done in France. Leblanc and collaborators (2005) estimated the average daily intake of mycotoxins in French population by analyzing 2,280 food samples. The samples were arranged in 456 composed samples according to different types of diet (children, adults, vegetarians). None of the tested samples showed aflatoxin concentration above the limit values set by the EU. The estimated daily intake of aflatoxins was between 0.12 and 0.34 ng kg^{-1} bw $day^{-1}$for adults, between 0.35 and 0.86 ng kg^{-1} bw day^{-1} for children and 0.4 and 0.9 ng kg^{-1}bw $day^{-1}$for vegetarians. The major intake of aflatoxins in adult population was estimated due the consumption of biscuits, cereals, eggs and products containing eggs, and in children due to the consumption of biscuits, cereals, chocolate and sweets. Similar results were obtained recently in a Spanish study (Cano-Sancho et al., 2013). The most exposed to aflatoxin intake were celiac sufferers with estimated average daily aflatoxin intake of 0.203 ng kg^{-1}bw day^{-1}, followed by the adolescents with intake of 0.189 ng kg^{-1}bw day^{-1}. In this research, the highest percentage of positive samples was found in red peppers, pistachios and peanuts. Another Spanish research on infant cereals pointed out that the consumers of organic cereals were more exposed to aflatoxins than the consumers of non-organic ones (Hernandez-Martinez and Navarro-Blasco, 2010). However, the estimated daily exposure to aflatoxins was in both groups below 1 ng kg^{-1}bw day^{-1}.The highest levels of aflatoxins were detected in cereals with cocoa, medium levels in those that were gluten-free or with dried fruit, while the lowest levels of aflatoxins were detected in milk or honey based cereals.

To minimize the health hazard due to the exposure to aflatoxins, JEFCA recommended that the daily intake of these toxins should be lower than 1 ng kg^{-1}bw day^{-1} (JEFCA 2001). In all researches conduced so far the estimated average daily intake of aflatoxins was below 1 ng kg^{-1}bw day^{-1} indicating that the health hazard due to the exposure to aflatoxins in major part of European population is not high.

Control and prevention of aflatoxin contamination: current state and future perspectives

The appropriate sampling protocols of materials for analysis are essential for minimizing the presence of aflatoxin contaminated food and feed on the market. In liquid media the sampling does not present particular problem, since the toxins are generally homogenously distributed. Grains and other solid media, usually, present less homogenous distribution of aflatoxin contamination. Often there are infected spots in the commodity, i.e. parts in which the environmental conditions were fa-

vorable for toxigenic fungus growth, and in the rest of the commodity the contamination is not present (Johansson et al., 2000; Whitaker et al., 2006). Therefore, the legislators have determined a suitable sampling protocol for each type of food and feedstuff. In all EU countries the samplings are done according the EU regulation 401/2006 and successive amendments. The most applied methods are ELISA and HPLC or GC-MS quantification of aflatoxins. ELISA is surely the most rapid method, but it can under or over-estimate the concentration of aflatoxins, and therefore it is normally used as a discriminator for samples that have to be analyzed by other methods. Quantification of aflatoxins in HPLC or GC-MS requires extraction and purification of samples, hence they are less rapid but more accurate and can indicate the concentration of toxin in a sample (Miraglia and Brera, 2005). There is an ongoing research in different laboratories on more simple and user-friendly devices which could alert a user (farmer, breeder, factory workers) when the concentration of aflatoxins in some food/feedstuff is near the allowed limits and when more accurate analyses are required before further processing.

Ever since the aflatoxins were discovered, the researches on efficient methods for the prevention of aflatoxin contaminations are numerous. Different approaches (i.e. chemical, biological, physical) have been studied. Anyhow, the first steps in the prevention of aflatoxin contamination in food and feed are identification of critical control points (CCPs) for aflatoxin contamination and their successive monitoring, HACCP (Magan, 2006). Due to the wide range of substrates and environmental conditions which favor the growth of aflatoxigenic fungi, different CCPs are present along the whole food/feed production chain. For example, some points in feed processing present a hazard for aflatoxin contamination due to moistening and heating which can create favorable environment for germination of *Aspergillus spp.* conidia eventually present on grains (Scudamore and Banks, 2004; Cavallucci et al., 2005).

Agriculture praxis

The two aflatoxigenic fungi, *A. flavus* and *A.parasiticus,* are able to colonize crops in pre and postharvest period. In both cases, stress conditions in plant (i.e. drought, insects, and physical damages of seeds) can improve the colonization (Gorman and Kang, 1991; Bottalico, 1998; Cavallucci et al., 2005). The “good agriculture praxis” is essential in protection against aflatoxin contamination. When possible, the drought stress should be avoided by irrigation, and eventually chemical treatments should be applied in periods of high risk. One of the technologies is based on anticipated harvesting to avoid the stress in plants, and then followed by rapid exsiccation of seeds (Reyneri et al., 2002; Gaspari et al., 2005a). Leaving the harvested crops on the soil for a longer time can increase the probability of infection by aflatoxinic fungi. Drying maize on the cob before shelling seems to be a good practice. Moreover, the damaging of seeds should be avoided in all phases of harvesting

and storage. Another problem present during the storage could be the attack from insects. Their activity could create moisture conditions that favor the growth of aflatoxigenic fungi in parts of stored seeds, hence the presence of insect during the storage should be kept to a minimum (Chulze, 2010).

Most of commercial maize hybrids are susceptible to *A. falvus* infections in the field. In the last decades, different hybrids resistant to *A. flavus* colonization were discovered (Campbell and White, 1995; Miedaner, 2004; Kelley et al., 2012). The selection of hybrids resistant to aflatoxigenic strains is one of promising strategies to control the presence of aflatoxins in food and feed. The resistance in maize is polygenic, even if the genes involved in the resistance has been cloned, a little is known about the mechanisms of this resistance. For the selection of resistant hybrids and new breeding markers need to be identified (Kelly et al., 2012, Cary et al., 2013). These markers could help the research of resistant cultivars in other species and eventually contribute to the creation of genetically modified organism (GMOs) resistant to aflatoxigenic fungi.

Use of chemical compounds

Use of chemicals, mainly with fungitoxic and fungistatic properties, is still the most used method of prevention (Miraglia and Brera, 2005; Passone et al., 2007). These products can have several side effects, like environmental pollution or selection of resistant fungal isolates, and therefore their application should be limited only to situations in which the risk of fungal contamination is high. Another group of chemical compounds used in the prevention of aflatoxin contamination are antioxidants (Nesci et al., 2003; Passone et al., 2007). The concentrations of some antioxidants, like butylated hydroxyanisole (BHA) or butylated hydroxytoluene (BHT), are limited by law due to their effects on humans and animals (European Food Safety Authority EFSA, 2011, 2012). Further problem with antioxidants can be their duration. Some experiments showed that BHA was effective in preventing aflatoxin synthesis on maize seeds contaminated by *A. flavus* for 10 days, but afterwards the amount of aflatoxins on treated seeds was higher than in the control, probably due to stress induced in fungal hyphae by BHA (Zjalic, 2006). Plant and fungal extracts, as well as natural antioxidants, (i.e. caffeic acid and other phenolic compounds) have shown ability to control aflatoxin synthesis (Fanelli et al., 2000; Razzachi et al., 2005; Joseph et al., 2005; Sanchez et al., 2005; Mohanlall e Odhav, 2006; Yu, 2012). Fungal polysaccharides provided a high (over 90%) and long aflatoxin inhibition both in *vitro* and in *vivo* experiments (Reverberi et al, 2005, 2008; Zjalic 2006; Zjalic et al 2006, 2012). These fungal polysaccharides, known for their beneficial effects on human and animal health (Zjalic et al., 2008), have also provided significant inhibition of other mycotoxins, such as ochratoxin A (Ricelli et al., 2002, 2005). Unfortunately,

the costs of the production of plant and fungal compounds are still too high for their commercial use in preventing mycotoxin presence in food and feed.

Bio-control agents

Utilization of bio-control agents in a challenge against aflatoxins is a promising field. The use of non aflatoxigenic strains of *A. flavus* as a competitor of toxigenic strains in the field has been proposed in Australia in peanut cultivation, and in the USA in cotton seed, corn and peanut fields (Dorner et al., 1999; Pitt, 2004; Cotty and Bayman, 1993; Cotty and Bathnagar, 1994). Several studies on a large-scale demonstrated the efficacy of atoxigenic strains in controling aflatoxin contamination in the field. Actually, two atoxigenic strains of *A. flavus* are in commercial use in the USA. Both commercial strains, Alfa-guard and AF37, have deleted parts of aflatoxin gene cluster and hence are unable to produce toxic compounds (Amaike and Keller, 2011). Recently, it has become apparent that many of mitosporic fungi undergo cryptic recombination via unobserved mechanisms and that asexual fungi are rare. Sexual recombination in *A. flavus* has been demonstrated, as well as the possibility of Afl- strains (atoxigenic) to become Afl+ strains (toxigenic) via crossing over during recombination (Fisher and Henk, 2012). Moreover, another research has pointed out that in the fields the ratio between two mating types of *A. flavus* necessary for recombination is 1:1, and that sexual recombination in nature occurs frequently (Moore et al., 2013). These facts could create problems in the application of atoxigenic strains in the control of aflatoxin production, and indicate that is necessary to take into account the possible recombination in researching new atoxigenic strains of *A. flavus* and *A. parasiticus*.

Decontamination

Detoxification of food/feedstuff is the only possibility when the contamination has already occurred. Some results in detoxification were obtained by peeling of the grains and, in peanut, by toasting (Scudamore and Banks, 2004). In some states, the use of chemical treatments, like ammonia, sodium bisulphite or calcium hydroxide, for decontamination of feedstuff is allowed. Besides eventual lowering of aflatoxin content, this treatments influence the nutritional value of feed by degrading important components like some essential amino acids (Piva et al., 1995). In animal production, aflatoxin-sequestering agents are used, especially in dairy cattle, to reduce aflatoxin M1 concentration in milk (Gaspari et al., 2005b; Jaynes, Zartman, 2011). The sequestering agents are normally clay or other mineral compounds used to adsorb aflatoxins in rumen. These agents are not specifically selective for aflatoxin adsorption so they could also adsorb useful compounds and decrease the quality of milk. Therefore the composition of meal seems to play an important role in their optimal functioning (Queiroz et al., 2011).

Microorganisms have not been applied yet in the control of aflatoxins on a commercial scale. There are several ongoing researches on their possible utilization, either for degradation of aflatoxins or their adsorption in animal digestive tract (Wu et al, 2009; Pizzolitto et al., 2012).

Conclusions

In the last fifty years, a lot has been known about the physiology and genetics of aflatoxins and about the control and prevention of their synthesis, but the "magic bullet" that solves all the problems has not been found yet, and it probably never will. Nevertheless, the improvements in the control and prevention of aflatoxin presence in food and feed stuff have enabled better food safety, especially in developed countries. Future researches should concentrate on developing new methods for early detection of aflatoxigenic fungi and aflatoxins in food and feed, and on developing environment friendly strategies for the prevention of their biosynthesis.

References

1. Abbas H.K., Weaver M.A., Zablotowitz R.M, Horn B.W. Shier W.T., 2005. Relationships between aflatoxin production and sclerotia formation among isolates of *Aspergillus* section Flavi from the Mississippi Delta. European J Plant Pathol 112: 283-287.
2. Adegoke G.O., Otumu E.J., Akanni A.O.,1994. Influence of grain quality, heat, and processing time on the reduction of aflatoxin B1 levels in 'tuwo' and 'ogi': two cereal-based products. Plant Foods Hum Nutr. 45:113-7.
3. Alpsoy L., Kiren A., Can S.N., Koprubasi A., 2013. Assessment of total aflatoxin level in red pepper obtained from Istambul. Toxicol Ind Health (epublished ahead of print)
4. Amaike S., Keller N.P., 2009. Distinct roles for VeA and LaeA in development and pathogenesis of *Aspergillus flavus.* Eukaryot Cell 8: 1051-1060.
5. Amaike S., Keller N.P., 2011. *Aspergillus flavus*. Ann rev Phytopathology 49: 107-133.
6. Andrade P.D., de Mello M.H., Franca J.A., Calades E.D., 2013. Aflatoxins in food products consumed in Brazil: a preliminary dietary risk assessment. Food Addit Contam Part A Chem Anal Control Expo Risk Assess 30: 127-136.
7. Angsurbharkon S., 2000. Aflatoxins. In: Mycotoxin prevention and control in food grains. R.L. Semple, A.S. Frio, P.A. Hicks and J.V. Lozare eds. UNDP/FAO publication (www.fao.org)
8. Azzam A.H., Gabal M.A., 1998. Aflatoxin and immunity in layer hens. Avian Pathol 27: 570-577.
9. Azziz-Baumgartner E., Lindlable K., Giesker L., Schurz Rogers H., Kiesxak S., Njapau H., Schleicher R., Mc Coy L.F., Misore A., DeCock K., Rubin C., Slusker L., 2005. Case-control study of an acute aflatoxicosis outbreak, Kenya 2004. Environ Health Persp 113: 1179-1783.
10. Bayrum O., Krappmann S., Ni M., Bok J.W., Helmstaedt K., 2008. The VelB/VeA/LaeA complex coordinates light signal with fungal development and secondary metabolism. Science 320: 1504-1506.

11. Bennett J.W., Klich M., 2003. Mycotoxins. Clin Microbiol Rev 16: 497 – 516.

12. Bennett J.W., Kronberg F.G., Goodman L.A., Seltman M.A., 1983. Isolation of an anthroquinone accumulating mutant of *Aspergillus parasiticus* and partial characterization by dry column chromatography. Mycologia 75: 202-208.

13. Bhatnagar D., Payne G.A., Cleveland T.E., Robens J.F., 2004. Mycotoxins: current issues in U.S.A. In*: Meeting the mycotoxins menace.* Barug D., van Egmond H.P., Lopez-Garcia R., van Osenbruggen W.A., Visconti A. (eds). Wageningen Academic Publishers, Wageningen pp 17-47

14. Bok J.W., Keller N.P., 2004. LaeA a regulator of secondary metabolism in *Aspergillus spp.* Eukaryot Cell 3: 527-535.

15. Bok J.W., Noordermeer D., Kale S.P., Keller N.P., 2006. Secondary metabolic gene cluster silencing in *Aspergillus nidulans*. MolMicrobiol 61: 1636-1645.

16. Bottalico A., 1998. Le micotossine nelle piante e nelle derrate. Italia Agricola 193-212.

17. Bouhired S., Webwer M., Kempf-Sontag A., Keller N.P., Hoffmeister D., 2007. Accurate prediction of the *Aspergillus nidulans* terrequinone gene cluster boundaries using the transcriptional regulator LaeA. Fungal Genet Biol 44: 1134-1145.

18. Brown D.W., Adams T.H., Keller N.P., 1996. *Aspergillus* has distinct fatty acid synthases for primary and secondary metabolism. Proc Natl Acad Sci *USA* 93: 14873-14877.

19. Burnside J.E., Sippel W.L, Forgacs J., Carll W.T., Atwood M.B., Doll E.R., 1957. A disease of swine and cattle caused by eating mouldy corn. II. Experimental production with pure cultures of moulds. Am J Vet Res. **18**: 817–824.

20. Burow G.B., Nesbitt T.C., Dunlap J., Keller N.P**.,** 1997. Seed lipoxygenase products modulate *Aspergillus* mycotoxin biosynthesis**.** Mol Plant-Microbe Interact 10**:** 380-387

21. Caloni F., Stammati A., Frigge G., De Angelis I., 2006. Aflatoxin M1 adsorption and cytotoxity on human intestinal in vitro model. Toxicona 47: 409-415.

22. Campbell K.W., White D.G., 1995. Inheritance of resistance to *Aspergillus* ear rot and aflatoxin in corn genotypes. Phytopatol 85: 886-896.

23. Cano-Sancho C., Sanchis V., Marin S., Ramos A.J., 2013. Occurrence and exposure assessment of aflatoxins in Catalonia (Spain). Food Chem Toxicol 51: 188-193.

24. Caporel L.R., 1976. Ergotism: The Satan Loosed in Salem? Science 192: 21-26.

25. Cardona T.D., Ilangantileke S.G., Noomhorm A., 2000. Aflatoxin research on grains in Asia. In: Mycotoxin prevention and control in food grains. R.L. Semple, A.S. Frio, P.A. Hicks and J.V. Lozare eds. UNDP/FAO publication (www.fao.org)

26. Cary J.W., Ehrlich K.C., 2006. Aflatoxigenicity in *Aspergillus:* molecular genetics, phylogenetic relationship and evolutionary implications. Mycopatho*l* 162: 167-177.

27. Cary J.W.,Ehrlich K.C., Kale S.P., Calvo A.M., Bhatnagar D., Cleveland T.E., 2006. Regulatory elements in aflatoxin biosynthesis. Mycotoxin Res 22. 105-109.

28. Cary J.W., Rajasekaran K., Brown R.L., Luo M., Chen Z.Y., Bathnagar D., 2013. Developing resistance to aflatoxin in maize and cottonseeds. Toxins 3: 678-696.

29. Cavallucci C., Villeri C., Terni L., Batistelli M., Della Monica M., Fioriti E., Migni M., Luneia M., 2005. Valutazione di alcuni parametri collegati allo svillupo della micotossine. In: Raporti ISTISAN 05/42: Micotossine in filiera agro-alimentare. (Miraglia M e Brera C. eds) ISSN, Roma pp 90-93

30. Chang P.K., Yu J., Bhatnagar D., Cleveland T.E., 1999. Repressor- AFLR interactions modulate aflatoxin biosynthesis in *Aspergillus parasiticus.* Mycopathologia 147: 105-112.

31. Chang P.K., Yu J., Bhatnagar D., Cleveland T.E., 2000. Characterization of the *Aspergillus parasiticus* major nitrogen regulatory gene, *areA*. Biochym Biophys Acta 1491: 263-266.

32. Chang P.K., 2004; lack of interaction between *aflR* and *aflJ* contributes to nonaflatoxigenicity of *Aspergillus sojae.* J Biotechnol 107: 245-253.

33. Chang P.K., Ehrlich K.C., Yu J., Bhatnagar D., Cleveland T.E., 1995. Increased expression of *Asapergillus parasiticus aflR* encoding sequence-specific DNA-binding protein, relives nitrate inhibition of aflatoxin biosynthesis. Appl Environ Microbiol 61: 2372-2377.

34. Chang P.K., Matsuhima K., Takahashi T., Yu J., Abe K., Bhatnaghar D., Yuan G.F., Koyoma Y., Cleveland T.E., 2007. Understanding nonaflatoxigenity of *Aspergillus sojae*: a windfall of aflatoxin biosynthesis research. App Microbiol Biotechnol 76: 977-984.

35. Chaves – Carballo, E., Ellefson, R.D., Gomez, M.R., 1976. An aflatoxin in liver of a patient with Reye – Johnson syndrome. Mayo Clin. Prroc. 51:48.

36. Christensen C.M.(ed), 1975. Moulds, Mushrooms and Mycotoxins. University of Minnesota Press, Minneapolis. pp 264.

37. Chulze S.N., 2010. Strategies to reduce mycotoxin levels in maize during storage: a review. Food Addit Contam Part A Chem Anal Control Expo Risk Assess 27: 651-657.

38. Cotty P.J, 1988. Aflatoxin and sclerotial production by *Aspergillus flavus*: Influence of pH. Phytopathol 78: 1250-1253.

39. Cotty P.J., Bayman P., 1993. Competitive extrusion of aflatoxigenic strain of *Aspergillus flavus* by an atoxigenic strain. Phytopathology 83: 1283-187.

40. Cotty P.J., Bathnagar D., 1994 Variability among atoxigenic *Aspergillus flavus* strains in ability to prevent aflatoxin contamination and production of aflatoxin biosynthetic pathway enzymes. Microbio 60: 2248-2251.

41. Crawford J.M., Vagstad A.L., Ehrlich K.C., Towsend C.A., 2006. Identification of a starter unit acyl-carrier protein transacylase domain in an alternative type I polyketide synthase. Proc Natl Sci USA 103: 16728-16733.rawford J.M., Vagstad A.L., Ehrlich K.C., Towsend C.A., 2008a. Starter unit specificity directs genome mining of polyketide synthase pathway in fungi. Bioorg Chem 36: 16-22.

42. Crawford J.M., Thomas P.M., Scheerer J.R., Vagstad A.L., Kelleher N.L., Towsend C.A., 2008b. Deconstruction of iterative multidomain polyketide synthase function. Science 320: 243-246.

43. Delaunay A., (Isnard A.D., Toledano M.B., 2000. H_2O_2 sensing through oxidation of the Yap1 transcriptional factor. Embo J 19: 5157-5166.

44. Dereszynski D.M., Center S.A., Randolph J.F., Brooks M.B., Hadden A.G. 2008. Clinical and clinic-pathologic features of dogs that consumed foodborne hepatotoxic aflatoxins: 72 cases 2005-2006. J. AM Vet Med Asoc 232: 1329-1337.

45. Dorner J.W., Cole R.J., Wicklow D.T., 1999. Aflatoxin reduction in corn through field application of competitive funghi. J. Food Protection 62: 650-656.

46. Du W., O'Braian G.R., Payne G.A., 2007. Function and regulation of *aflJ* in the accumulation of aflatoxin early pathway intermediate in *Aspergillus parasiticus.* Food Addit Contam 24: 1043-1050.

47. Duran R.M., Cary J.W., Calvo A.M., 2007. Production of cyclopiazonic acid, aflaterm and aflatoxin by *Aspergillus flavus* is regulated by *veA*, a gene necessary for sclerotia formation. Appl Microbiol Biotechnol 73: 1158-1168.

48. Dutton M.F., 1988. Enzymes and aflatoxin synthesis. Microbiol Rev 52: 274-295.

49. Dvorachova, L., Zilkova, J., Bredsky, F., Cerman, J., 1972. Aflatoxin and liver damage with encephalopathy. Shornik redeckych praci Lekarske fakulty uity K U V Hradci Kralove 15:521.

50. Eaton D., Gallhager E.P., 1994. Mechanism of aflatoxin Carcinogenesis, Annu Rev Pharmacol Toxicol. 34:135-72.

51. Eaton D., Groopman J. (eds), 1998. The toxicology of aflatoxins: human health, veterinary and agricultural significance. Academic Pres, San Diego, pp 552.

52. EFSA, 2011. Scientific opinion on the re-evaluation of butylated hydroxyanisole BHA (E320) as a food additive. EFSA Journal 9: 2392

53. EFSA2012. Scientific opinion on the re-evaluation of butylated hydroxytoluene BHT (E321) as a food additive. EFSA Journal 10: 2588.

54. Ehrlich K.C., Mack B.M., Wei Q., Li P., Roze L.V., Dazzo F., Carry J.W., Bhatnagar D., Linz J.E., 2012. Association with AflR in endosome reveals new function for AflJ in aflatoxin biosynthesis. Toxins (Basel): 1582-1600.

55. Ehrlich K.C., Montalbano B.G., Cary J.W., 1999. Binding of the C6-zinc cluster protein AFLR to the promoters of aflatoxin pathway biosynthesis genes in *Aspergillus parasiticus.* Gene 230: 249-257.

56. El-Kady I., El- Maraghby S.., Zihri A.N., 1994. Mycotoxin producing potential of some isolates of *Aspergillus flavus* and *Eurotium* groups from meat products. Microbiol Res 149: 297-307.

57. El-Zawahari M.M, Morad M.M., Khishin A.F., 1990. Mutagenic effect of aflatoxin G1 in comparison with B1. J Environ Pathol Toxicol Oncol 10:45-51.

58. Etam R., 1996. Growth and aflatoxin B1 production on olives and olive paste by moulds isolated from 'Turkish-style' natural black olives in brine. Int J Food Microbiol 32: 217-223.

59. Fabbri A.A., Fanelli C., Panfili G., Passi S., Fasella P., 1983. Lipoperoxidation and aflatoxin biosynthesis by *Aspergillus parasiticus* and *A.flavus.* J Gen Microbiol 129:3447-3452.

60. Fanelli C., Fabbri A.A., Finotti E., Panfili F., Passi S., 1983**.** Cerulenin and tetrahydrocerulenin: stimulating factors of aflatoxin biosynthesis. Trans Br Mycol Soc 81: 201-204.

61. Fanelli C., Fabbri A.A., Pieretti S., Finotti E., Passi S., 1985. Effect of different antioxidants and free radical scavengers on aflatoxin production. Mycotox Res1: 65-69.

62. Fanelli C., Tasca V., Ricelli A., Reverberi M., Zjalic S., Finotti E., Fabbri A.A., 2000. Inhibiting effects of medicinal mushroom *Lentinus edodes* (Berk.) Singer (Agaricomycetdeae) on aflatoxin production by *Aspergillus parasiticus* Speare. Int J Med Mushrooms, 2: 88-95.

63. Fink-Gremmels J., 1999. Mycotoxins: their implications for human and animal health. Vet Q 21: 114-120.

64. Fisher R., 2008. Sex and poison in the dark. Science 320: 1430-1431.

65. Fisher M.C., Henk D.A., 2012. Sex, drugs and recombination: the wild life of *Aspergillus*. Mol Ecol 21: 130-1306.

66. Fishle W., Tseng B.S., Dorman H.L., Ueberheide B.M., Garcia B.A., Schabanowitz J., Hunt D.F., Funabiki H., Allis C.D., 2005. Regulation of HP-1 chromatin by histone H3 metilation and phosphorilation. Nature 438: 1116-1122.

67. Flaherty J.E., Payne G.A., 1997. Overexpression of *aflR* leads to upregulation of pathway gene expression and increased aflatoxin production in *Aspergillus flavus.* Appl Environ Microbiol 59: 3995-4000.

68. Frisvad J.C., Houbraken J., Samson R.A., 1999. *Aspergillus* species and aflatoxin production: a reappraisal. In: Food Microbiology and Food Safety into the New Millenium. Tuijtelaars A.C.J., Samson R.A., Notermans S. (eds), Foundation Food Micro, Zeist: 125-126.

69. Frisvad J.C., Samson R.A., 2004. *Emericella venezuelensis*, a new species with stellate ascospores producing sterigmatocystin and aflatoxin B1. System Appl Microbiol 27: 672-680.

70. Frisvad J.C., Skouboe P., Samson R.A., 2005. Taxonomic comparison of three different groups of aflatoxin producers and a new efficient producer of aflatoxin B1, sterigmatocystin and 3-O-methylsterigmatocystin, *Aspergillus rambellii* sp. nov. Syst Appl Microbiol 28: 442-453.

71. Frisvad J.S., Smedsgaard J., Samson R.A., 2004. *Emericella astellata*, a new producer of aflatoxin B1, B2, and sterigmatocystin. Lett Appl Microbiol 38: 440-445.

72. Garland T., Reagor J., 2001. Chronic canine aflatoxicosis and management of epidemic. In: Mycotoxin and Phytotoxins in perspectives at the turn of the millennium. DeKoe W., Samson R., van Egmond (eds). Ponsen and Looven, Wageningen Nl pp 231-236.

73. Gaspari F., Bortolotti M., Cinti F., 2005a. Influenza dell'umidità alla raccolta e della sosta in attesa dell'essiccamento sulla contaminazione con aflatossine della granella di mais. In: Raporti ISTISAN 05/42: Micotossine in filiera agro-alimentare. (Miraglia M. e Brera C. eds) ISSN, Roma pp 206-209.

74. Gaspari F., Piccaglia R., Corsari A., Tampieri A., 2005b. Influenza del sequestrante Atox ^{Tm} – bentonite sulla riduzione di aflatossina M1 in latte. In: Raporti ISTISAN 05/42: Micotossine in filiera agro-alimentare. (Miraglia M. e Brera C. eds) ISSN, Roma pp 217-219.

75. Georgianna R.D., Payne G.A., 2009. Genetic regulation of aflatoxin biosynthesis: From gene to genome. Fungal Genet Biol 46: 113-125.

76. Ghitakou S., Koutras K., Kanellou E., Markaki P., 2006. Study of aflatoxin B1 and ochratoxin A production by natural microflora and *Aspergillus parasiticus* in black and green olives of Greek origin. *Food Microbiol* 23: 612-621.

77. Goldman R., Shilds P.G., 2003. Food Mutagens. *J Nutr* 133(S3): 965S-973S.

78. Gorman D.P., Kang M.S., 1991. Preharvest aflatoxin contamination in maize: resistance and genetics. Plant Breeding 107: 1-10.

79. Haouet M.N. & Altissimi M.S., 2003. Micotossine negli alimenti e micotossicosi animale e umana- Elaborazione dei dati ottenuti dal 1992 al 1998. Webzine- Sanità pubblica veterinaria vol. IV numero 19 (htp:www. pg.izs.it/webzine.html)

80. Hegazy S.M., Azzam A., Gabal M.A., 1991. Interaction of naturally occurring aflatoxins in poultry feed and immunization against fowl cholera. Polut Sci 70: 2425-2428.

81. Hirota T., Lipp J.J., Toh B.H., Peters J.M., 2005. Histone H3 serine 10 phosphorylation by Aurora B causes dissociation from heterochromatin. Nature 438: 1176-1180.

82. Hong S.Y., Roze L.V., Linz J.E., 2013. Oxidative stress-related factors in regulation of secondary metabolism. Toxins 5: 683-702.

83. Horn B.W., Greene R.L., Sorensen R.B., Blankenship P.D., Dorner J.W., 2001. Conidial movement of nontoxigenic *Aspergillus flavus* and *A. parasiticus* in peanut fields following application to the soil. Mycopathologia 151: 81-92.

84. Horowitz B.S., Scott J.B., Bhaheetharan J., Sharpe W.C., Mlide L., 2009. Oxygenase coordination is required for morphological transition and the host-fungal interaction of *A. flavus.* Mol Plant-Microbe Interact 22: 882-894.

85. IARC, 2013. Agents classified by IARC monographs, Volumens 1-108. (http://monographs.iarc.fr/ENG/Cclassification/ClassificatioAlphaOrder.pdf.)

86. Jayashree T., Subramanyam C., 2000. Oxidative stress is a prerequisite for aflatoxin production by *Aspergillus parasiticus.* Frre Rad Biol med 10: 981-985.

87. Jaynes W.F., Zartman R.E., 2011. Aflatoxin toxicity reduction by enhanced binding to surface-modified clay additives. Toxins (Basel) 3: 551-565.

88. JEFCA. 2001. Safety evaluation of certain mycotoxins in food. 56th Meeting of joint FAO/WHO Expert Comitee on Food Additives. WHO Geneva. Food Additives Series 47.

89. Johansson A.S., WhitakerT.B., Hagler W.M., Giesbrecht F.G., Young J.H., Bowman D.T., 2000. Testing shelled corn for aflatoxin, Part I: estimation of variance components. J AOAC Int 83:1264-1269.

90. Joseph G.S., Jayaprakasha G.K., Selvi A.T., Jena B.S., Sakariah K.K., 2005. Antiaflatoxigenic and antioxidant activities of *Garcinia* extracts. Int J Food Microbiol 101:153-160.

91. Kale S.P., Cary J.W., Hollins N., Wilkinson J.R., Bhatnagar D., Yu J., Cleveland T.E., Bennett J.W., 2007. Analysis of aflatoxin regulatory factors in serial transfer-induced non aflatoxigenic *Aspergillus parasiticus.* Food Addit Contam 24: 1061-1069.

92. Kale S.P., Milde L., Trapp M.K., Frisvad J.C.m Keller N.P., Bok J.W., 2008. Requirement of LaeA for secondary metabolism and sclrotial production in *Aspergillus flavus.* Fungal Genet Biol 45: 1422-1429.

93. Karenlampi S.O., 1987. Mechanism of cytotossicity of aflatoxin B1: role of cytochrome P1-450. Biochem Biophys Res Commun 145: 854-860.

94. Keller N.P., Turner G., Bennett J.W., 2005. Fungal secondary metabolism- from biochemistry to genomics. Nature Rew 3:937-947.

95. Kelley, R.Y., Williams P.W., Myrole E.J., Boykin D.L., Harper J.W., Windham G.L., Ankala A., Shan X., 2012. Identification of maize genes associated with host plant resistance or susceptibility to *Aspergillus flavus* infection and aflatoxin accumulation. PLoS ONE : e36892.

96. Khlangwiset P., Shephard G.S., Wu F., 2011. Aflatoxins and growth impairment: a review. Crit Rev Toxicol 41: 740-755.

97. Klich M.A., Pitt, J.1., 1985. The theory and practice of distinguishing species of the *Aspergilus flavus* group. In: *Advances in Penicillium and Aspergillus Systematics*, R.A. Samson and J.l. Pitt (eds). Plenum Press, New York. pp. 211 -220.

98. Klich, M.A. and Pitt, J.l., 1988. Differentiation of *Aspergillus flavus* from *A. parasiticus* and other closely related species. Trans Br Mycol Soc 91: 99-108.

99. Kurtzman C.P., Horn B.W., Heseltine C.W., 1987. *Aspergillus nomius* a new aflatoxin-producing species related to *Aspergillus flavus* and *Aspergillus parasiticus*. Antonie van Leeuwenhoek 53:147-158.

100. Leblanc J.C., Tard A., Volatier J.L., Verger P., 2005. Estimated dietary exposure to principal food micotoxins from the first French total diet study. Food Add Contam 22:652-672.

101. Lereau M., Gouas D., Villar S., Besaratinia A., Hautefeuille A., Berthilon P et al., 2012. Interaction between hepatitis B virus and aflatoxin B(1): effects on p53 induction in HepaRG cells. J Gen Virol 93: 640-650.

102. Lewis L., Onsongo M., Njapau H., Shurzogers H., Luber G., Kieszak S., Nyamongo J., Backer L., Dahiye A.M., Misore A., DeCock K., Rubin C., 2005. Aflatoxin contamination of commercial maize products during an outbreak of acute aflatoxicosis in eastern and central Kenya. Environ Health Persp 113: 1763-1778.

103. Lopez-Huertas E., Charlton W.L., Johnson B., Graham I.A., Baker A., 2000. Stress induces peroxisome biogenesis genes. Embo J 19: 6770-6777.

104. Magan N., 2006.Mycotoxin contamination of food in Europe: early detection and prevention. Mycopathol 162: 245-253.

105. Maggio-Hall L.A., Wilson R.A., Keller N.P., 2005. Fundamental contribution of beta-oxidation to polyketide mycotoxin productio*n in planta.* Mol Plant-Micorb Interact 18: 783-793.

106. Malby M., Mankotia M., Cavlovic P., Tam J., Wong L., Pantazpoulos P., Calway P., Scott P.M., 2005. Survey of aflatoxins in beer sold in Canada. Food Addit Contam 22: 1252- 1257.

107. Matossian M.K.(ed), 1989, Poisons of the Past: Molds, Epidemics, and History, New Haven & London, Yale University. Pp208

108. Matossian M.K., 1984. Mold poisoning and population growth in England and France, 1750-1850. J Econ Hist. 1984; 44(3):669-686.

109. Matossian M.K., 1986. Did mycotoxins play a role in bubonic plague epidemics? Perspect Bio Med 29: 244-256.

110. Miedaner T., 2004. Plant breeding as a tool for reducing mycotoxins in cereals. In: Meeting the mycotoxins menace. Baroug D., van Egmond H.P., Lopez-Garcia R., van Osenbruggen W.A., Visconti A. (eds) Wageningen Academic Publishers, Wageningen, pp 89-109.

111. Mita G., Fasano P., de Domenico S., Perrone G., Epifani F., Iannacone R., Casey R., Santino A., 2007. 9-lipoxygenase is involved in the almond/*Aspergillus carbonarius* interaction. J Exp Bot 58: 1803-1811.

112. Miraglia M., Brera C., 2005. Problematiche legate alla fase analitica nella determinazione delle micotossine nei prodotti alimentari. In: Raporti ISTISAN 05/42: Micotossine in filiera agro-alimentare. (Miraglia M e Brera C. eds) ISSN, Roma pp 267-272.

113. Mohanlall V., Odhav B., 2006. Biocontrol of aflatoxins B1, B2, G1, G2, and fumonisin B1 with 6,7-dimethoxycoumarin, a phytoalexin from *Citrus sinensis*. Food Prot 69: 2224-2229.

114. Mooney J.L., Yagr L.N., 1990. Light is required for conidiation in *Aspergillus nidulans.* Genes Dev 4: 1473-1482.

115. Moore G., Elliot J.L., Singh R., Horn B.W., Dorner J.W., Stone E.A., Chulze S.N., Barros G.G., Naik M.K., Wright G.C., Hell K., Carbone I., 2013. Sexuality generates diversity in aflatoxin gene cluster: evidence on a global scale. PLOS Pathogens 9: e1003574..

116. Neal G.E., Eaton D.L., Judah D.J., Verma A., 1998. Metabolism and toxicity of aflatoxin M1 and B1 in human-derived in vitro system. Toxicol Appl Pharmacol 151: 152-158.

117. Nesci A., Rodriguez M., Etcheverry M., 2003. Control of *Aspergillus* growth and aflatoxin production using antioxidants at different conditions of water activity and pH. J Appl Microbiol 95: 279-287.

118. Nesci A., Montemarani A., Etcheverry M., 2011. Assessment of mycoflora and infestation of insects, vector of *Aspergillus* section Flavi, in stored peanut from Argentina. Mycol Res 27: 5-12.

119. Ni X., Wilson J.P., Buntin G.D., Guo B., Krakowsky M.D., Lee R.D., Cottrell T.E., Scully B.T., Huffaker A., Schmelz E.A., 2011. Spatial patterns of aflatoxin levels in relation to ear-feeding insect damage in pre-harvest corn. Toxins 3: 920-931.

120. O'Brian G.R., Georgianna D.R., Wilkinson J.R., Yu J., Abbas H.K., Bhatnagar D., Cleveland T.E., Nierman W.C., Payne G.A., 2007. The effect of elevate temperature on gene transcription and aflatoxin biosynthesis. Mycologia 99: 232-239.

121. Passone M.A., Resnik S., Etchevery M.G., 2007. Antiaflatoxigenic property of food grade antioxidants under different conditions of water activity in peanut grains. Int J Food Microbiol 118: 8-14.

122. Payne G.A., Hagler W.M., 1983. Effect of specific aminoacids on growth and aflatoxin production by *Aspergillus parasiticus* and *A. flavus* in defined media. Appl Environ Microbiol 171: 1539-1545.

123. Payne G.A., Brown M.B., 1998. Genetics and physiology of aflatoxin synthesis. Appl Environ Microbiol 54: 2096-2100.

124. Penalva M.A., Tiburn J., Bignell E., Arst H.N. Jr., 2008. Ambient pH gene regulation in fungi: making connections. Trends Microbiol 16: 291-300.

125. Perrin R.M., Fedorova N.D., Bok J.W., Cramer R.A., Wortman J.R., Kim H.S., Nierman W.C., Keller N.P., 2007. Transcriptional regulation of chemical diversity in *Aspergillus fumigatus* by Lae. PLoS Pathog 3, e50.

126. Piccaglia R., Grandi S., Canever A., Transerici E., 2005. Indagine sulla presenza di aflatossine in mangimi prodotti da diverse aziende. In: Raporti ISTISAN 05/42: Micotossine in filiera agro-alimentare. (Miraglia M e Brera C. eds) ISSN, Roma pp 215-216.

127. Pitt J.l., 2000. Introduction to Mycotoxins. In: Mycotoxin prevention and control in food grains**.** R.L. Semple, A.S. Frio, P.A. Hicks and J.V. Lozare eds. UNDP/FAO publication (www.fao.org)

128. Pitt J.I., 2004. Biocontrol of aflatoxins in peanuts. In*:* Meeting the mycotoxins menace. Baroug D., van Egmond H.P., Lopez-Garcia R., van Osenbruggen W.A., Visconti A. (eds) Wageningen Academic Publishers, Wageningen, pp143-154.

129. Piva G., Galvano F., Pietri A., Piva A., 1995. Detoxification methods of aflatoxins. A review. Nutr Res 15: 767-776.

130. Pizzolitto R.P., Armando M.R., Combina M., Cavaglieri L.R., Balcero A.M., Salvano M.A., 2012. Evaluation of *Saccharomyces cervisiae* strains as probiotic agent with aflatoxin B1 adsorption ability for use in poultry feedstuff. J Environ Sci Health 47: 933-941.

131. Price M.S., Nierman W.C., Kim H.S., Pitchard B., Jacobus C.A., Bhatnagar D., Cleveland T.E., Payne G.A., 2006. The aflatoxin pathway regulator AflR induces gene transcription inside and outside of the aflatoxin biosynthetic cluster. FEMS Mycrobiol Lett 255: 275-279.

132. Price W.D., Lovell R.A., McCheseney D.G., 1993. Naturally occurring toxins in feedstuffs:center for veterinary medicine perspective. *J Anim Sci* 71: 2556-2562.

133. Querioz O.C.M, Han J.H., Staples C.R., Adesogan A.T., 2011. Effect of adding mycotoxin-sequestering agent on milk aflatoxin M1 concentration and the performance and immune response of dairy cattle fed an aflatoxin B1-contamined diet. J Diary Sci 95: 5901-5908.

134. Razzachi A.M., Allameh A., Tiraihi T., Shams-Ghahfarohki M, GhorbanianM., 2005. Morphological alterations in toxigenic *Aspergillus parasiticus* exposed to neem (*Azadirachta indica*) leaf and seed aqueous extracts. Mycopathol 159: 565-570.

135. Reddy T.V., Viswanthan L., Venkitasubramanian T.A., 1979. Factors affecting aflatoxin production by *Aspergillus parasiticus.* J Gen Microbiol 114: 409-413.

136. Reverberi M., Fabbri A.A., Zjalic S., Ricelli A., Punelli F.,Fanelli C., 2005. Antioxidant enzymes stimulation in *Aspergillus parasiticus* by *Lentinula edodes* inhibits aflatoxin production. App Microbiol Biotechnol 69: 207-215.

137. Reverberi M., Zjalic S., Ricelli A., Fabbri A.A., Fanelli C., 2006. Oxidant-antioxidant balance in *Aspergillus parasiticus* affects aflatoxin biosynthesis. Mycotoxin Res 22: 39-47.

138. Reverberi M., Zjalic S., Punelli F., Ricelli A., Fabbri A.A., Fanelli C., 2007. ApyapA affects aflatoxin biosynthesis during *Aspergillus parasiticus* growth in maize seeds. Food Add Contam 24: 1070-1075.

139. Reverberi M., Zjalic S., Ricelli A., Punelli F., Camera E., Fanelli C., Fabbri A.A., 2008. Modulation of antioxidant defense in *Aspergillus parasiticus* is involved in aflatoxin biosynthesis: a role for th ApyapA gene. Eukaryotic Cell 7: 988-1000.

140. Reverberi M., Ricelli A., Zjalic S., Fabbri A.A., Fanelli C., 2010. Natural functions of mycotoxins and control of their biosynthesis in fungi. App Microbiol Biotechnol 87: 899-911.

141. Reverberi M., Zjalic S., Ricelli A., Di Meo C., Scarpari M., Fanelli C., Fabbri A.A., 2011. Mushroom versus fungi: natural compounds from *Lentinula edodes* inhibit aflatoxin biosynthesis by *Aspergillus parasiticus.* World Mycotoxin J 4: 217-224.

142. Reverberi M., Punelli M., Carry A., Zjalic S., Cardinali G., Pinzari F., Payne G.A., Fabbri A.A., Fanelli C., 2012. How peroxisomes affect aflatoxin biosynhesis in *Aspergillus flavus.* PLoS One 7: e48097.

143. Reyes-Dominguez Y., Bok J.W., Berger H., Schwab E.K., Basheer A., 2010. Heterochromatic marks are associated with the repression of secondary metabolism cluster in *Aspergillus nidulans.* Mol Microbiol 76: 1376-1386.

144. Ricelli A., Fabbri A.A., Nisini-Trionfetti P., Reverberi M., Zjalic S., Fanelli C., 2002. Inhibiting effect of different edible and medicinal mushrooms on the growth of two ochratoxigenic microfungi. Int J Med Mushroom 4: 171-177.

145. Riceli A., Zjalic S., Reverberi M., Taddei F., Fabbri A.A., Fanelli C., 2005. Resveratrol, *Lentinula edodes* and BHA: novel opportunities in the prevention of fungal growth and mycotoxin production. In: Modern fungicides and antifungal compounds. Dehne H.W., Gisi U., Kuck K.H., Russel P.E., Lyr H. (eds). British crop production council press, GB. Pp 329-340.

146. Rodrigues I., Naehrer K., 2012. A three-year survey on the worldwide occurrence of mycotoxins in feedstuffs and feed. Toxins 4: 663-675.

147. Roll R., Matthiaschk G., Korte A., 1990. Embryotoxicity and mutagenicity of mycotoxins. J. Environ Pathol Toxicol Oncol 10: 1-7.

148. Rosinberg, H. 1972. Diagnostiche Moglickeiten zum Nachweis von Aflatoxin Vorgftungen. Zentralbe Bakeriol. Parasitenkd. Infektionskr Hyg. Ast. 1. 220-252.

149. Roussos S, Zaonia N., Salih G., Tantaoui-Erlaki A., Lamari K., Cheheb M., Hassouni H., Verhe F., Perraud-Gaime I., Augur C., Ismail-Alaoui M., 2006. Characterization of filamentous fungi isolated from Moroccan olive and olive cake: toxinogenic potential of *Aspergillus* strains. Mol Nutr Food Res 50:500-506.

150. Roze L.V., Arthur A.R., Calvo A.,M., Linz J.E., 2007a. The initiation and pattern of spread of histone H4 acetylation parallel the order of transcriptional activation of genes in the aflatoxin cluster. Mol Microbiol 66: 713-726.

151. Roze L.V., Beaudry R.M., Arthur A.R., Hong S.Y., Chanda A., Linz J.E., 2007b. *Aspergillus* volatiles regulate aflatoxin synthesis and sexual sporulation in *Aspergillus parasiticus.* Appl Environ Microbiol 73: 7268-7276.

152. Saito M., Tsuruta O., 1993. A new variety of *Aspergillus flavus* from tropical soil in Thailand and its aflatoxin productivity. Proc Jpn Assoc Mycotoxicol 37: 31-36.

153. Sanchez E., Heredia N., Garcia S., 2005. Inhibition of growth and mycotoxin production of *Aspergillus flavus* and *Aspergillus parasiticus* by extracts of Agave species. Int J Food Microbio. 98: 271-279.

154. Sargent, K., Carnahan, R.B.A., Allcroft, R. 1963. Toxic products in groundnuts – chemistry and origin. Chemy Ind. 1963: 55-61.

155. SCOOP (Scientific Cooperation on Questions Relating to Food Projects), 1996. Working document in support of a SCF risk assessment of aflatoxin scientific cooperation on questions related to food projects, task 3.2.1(SCOOP/CNTM/1), Task coordinator-UK.

156. Scudamore K.A., Banks J.N., 2004. The fate of mycotoxins during cereal processing. In: Meeting the mycotoxins menace. Baroug D., van Egmond H.P., Lopez-Garcia R., van Osenbruggen W.A., Visconti A. (eds) Wageningen Academic Publishers, Wageningen, pp165-182.

157. Selvig K., Alspaugh A.J., 2011. pH response pathways in fungi: Adapting to host-derived and environmental signals. Mycrobiol 39: 249-256.

158. Serck- Hanssen, A., 1970. Aflatoxin – induced fatal hepatitis?. A case report from Uganda. Arch Environ Health. 20:729.

159. Shank R.C., Bourgeois C.H., Keschamras N., Chandavimol P., 1971. Aflatoxin and autopsy specimens from Thai children with an acute disease of unknown aetiology. Food Cosmet. Toxicol. 9: 501-507.

160. Shivanchandra S.B., Sahl R.L., Singh S.D., Kataria J.M., Manimaran K., 2003. Immunosuppression in broiler chicks fed aflatoxin and inoculated with fowl adenovirus serotype-4(FAV4) associated with hydropericardium syndrome. Vet Res Comun 27: 39-51.

161. Streit E., Schatzmayr G., Tassis P., Tzika E., Marin D., Taranu I., Tabuc C., Nicolau A., Aprodu I., Puel O., Oswald I., 2012. Current situation of mycotoxins contamination and co-occurrence in animal feed – focus on Europe. Toxins 4: 788-809.

162. Smela M.E, Curier S.S., Bailey E.A., Essingmann J.M., 2001. The chemistry and biology of aflatoxin B1. Carcinogenesis. 22: 535-545.

163. Smith L.E., Stoltzfus R.J., Prendergast A., 2012. Food chain mixotoxin exposure, gut health, and impaired growth: a conceptual framework. Adv Nutr 3: 526-531.

164. Soares C., Rodrigez P., Peterson S.W., Lima N., Venancio A., 2012. Three new species of *Aspergillus* section Flavi isolated from almonds and maize in Portugal. Mycologia 104: 682-697.

165. Sun Z.M., Qi Z.T, 1991. A new aflatoxin producing species of sect. Flavi of *Aspergillus*. Acta Mycol Sin 10: 22-26.

166. Tessari E.N., Oliveira C.A., Cardoso A.L., Ledoux D.R., Rottinghaus G.E., 2006. Effects of aflatoxin B1 and fumonisin B1 on body weight, antibody titres and histology of broiler chicks. Br Poult Sci 47: 357-364.

167. Toone W.M., Jones N., 1998. Stress activated signalling pathway in yeast. Genes Cells 3: 485-498.

168. Trucksess M.W., Scott P.M., 2008. Mycotoxins in botanicals and dried fruits: A review. Food Addit Contam Part A 25: 181-192.

169. Tsitsigiannis D.I., Kunze S., Willis D.K., Feussner I., Keller N.P., 2005. *Aspergillus* infection inhibits the expression of peanut 13S-HPODE–forming lypoxygenases. Mol Plant-Microb Interact 18: 1081-1089.

170. Tsitsigiannis D.I., Keller N.P., 2007. Oxylipins as developmental and host-communicating signals. Trends Microbiol 15: 109-118.

171. Vallone L., Boscariol D., Dragoni I., 2006. Aflatoxins in organic milk and dairy products. Vet Res Comm 30: 369-370.

172. Van Waalbeek W., Scott P.M., Thatcher F.S., 1968. Mycotoxins from some food-borne fungi. Can J MicrobiolI 14:131-137.

173. Varga J., Frisvad J.C., Samson R.A., 2011. Two new aflatoxin producing species, and an overview of *Aspergillus* section Flavi. Stud Mycol 69: 57-80.

174. Wangikar P.B., Dwivedi P., Sinha N., Sharma A.K., Telang A.G., 2005. Effects of aflatoxin B1 on embryo fetal development in rabbits. Food Chem Toxicol 43: 607-615.

175. WangikarP.B., Dwivedi P., Sinha N., SharmaA.K., Telang A.G., 2005. Effects of aflatoxin B1 on embryo fetal development in rabbits. Food Chem Toxicol 43: 607-615.

176. Whitaker T.B., Slate A.B., Jacobs M., Hurley J.M., Adams J.G., Giesbrecht F.G., 2006. Sampling almonds for aflatoxin, part I: estimation of uncertainty associated with sampling, sample preparation, and analysis. J AOC Int 89: 1027-1034

177. Whitlow L.W, Hagler W.M., 2002. Mycotoxins in feeds. Feedstufs 74: 1-9.

178. Wicklow D.T., Wilson D.M., Nelsen T.C., 1993. Survival of *Aspergillus flavus* sclerotia and conidia buried in soil in Illinois. Phytopathology 83: 1141-1147.

179. Woloshuk C.P., Foutz K.R., Brewer J.F., Bhatnagar D., Cleveland T.E., Payne G.A., 1994. Molecular characterization of *aflR*, a regulatory locus for aflatoxin biosynthesis. Appl Environ Microbiol 59: 156-162.

180. Woolf A., 2000. Witchcraft or mycotoxins? The Salem Witch trials. J Toxicol Clin Toxicol 38: 457-460.

181. Wu Q., Jezkova A., Pavlikova L., Dohnal V., Kuca K., 2011. Biological degradation of aflatoxins. Drug Metab Rev 41: 1-7.

182. Yabe K., Nakajima H., 2004. Enzyme reactions and genes in aflatoxin biosynthesis. Appl Microbiol Biotechnol 64: 745-755.

183. Yazdanpanah H., Mohammadi T., Abouhossain., Cheraghali A.M., 2005. Effect of roasting on degradation of aflatoxins in contaminated pistachio nuts. Food Chem Toxicol. 43:1135-9.

184. Yijang Y., Jolly P.E., Ellis W.O., Wang J.S., Phillips T.D., Williams J.H., 2006. Aflatoxin B1 albumin adduct levels and cellular immune status in Ghanaians. Int Immunol 17: 806-814.

185. Yu J., Butchko R.A., Fernandes M., Keller N.P., Leonard T.J., Adams T.H., 1996. Conservation of structure and function of the aflatoxin regulatory gene aflR from *Aspergillus nidulans* and *A. flavus.* Curr Gen 29:549-555.

186. Yu J.,Chang P.K., Ehrlich K.C., Cary J.W., Bhatnagar D., Cleveland T.E., Payne G.A., Linz J.E., Woloshuk C.P., Bennet J.W., 2004. Clustered pathway genes in aflatoxin synthesis. App Environ Microbiol 70: 1253-1262.

187. Yu J., Fedorova N.D., Montalbano B.G., Bhatnagar D., Cleveland T.E., Bennett J.W., Nierman W.C., 2011. Tight control of mycotoxin biosynthesis gene expression in *Aspergillus flavus* by temperature as revealed by RNA-Seq. FEMS Microbiol Lett 322:145-149.

188. Yu Y., 2012. Current understanding on aflatoxin biosynthesis and future perspectives in reducing aflatoxin contamination. Toxin 4: 1024-1057.

189. Zjalic S., 2006. Utilizzo di basidiomiceti nella lotta alle aflatossine prodotte da *Aspergillus parasiticus* Speare. PhD thesis. University La Sapinza, Rome. Pp 152.

190. Zjalic. S., Reverberi M., Ricelli A., Granito V.M., Fabbri A.A., Fanelli C., 2006. *Trametes versicolor*: A possible tool for aflatoxin control. Int J Food Microbiol 107: 243-249

191. Zjalic S., Reverberi M., Ricelli A., Fabbri A.A., Fanelli C., 2008. Medicinal mushrooms. In: Microbe biotechnology in agriculture. Ray R.C., Ward O.P (eds), Enfield Science Publisher, USA. pp 299-339.

192. Zjalic S., Reverberi M., Ricelli A., Scarpari M., Fabbri A.A., Fanelli C., 2012. Control of mycotoxins in feed by novel more environmental friendly strategies. In: Animal farming and environmental interactions in the Mediterranean region. EAAP publications 131. Casasus I., Rogosic J., Rosati A., Stokovic I, Gabina D. (eds). Wageningen academic publishers, NL. Pp 186-198.

Development of Integrated Bioprocess for Ethanol Production from Sugar Beet

Mladen Pavlečić, Mirela Ivančić Šantek, Predrag Horvat and Božidar Šantek*

Preliminary Communication

Department of Biochemical Engineering, Faculty of Food Technology and Biotechnology, University of Zagreb, Pierottijeva 6, 10000 Zagreb, Croatia

Ethanol production (as a biofuel) from renewable raw materials is still more expensive than gasoline production from fossil oil. Therefore, integration of different technological stages into one single stage could result in a more cost-effective and energy saving bioprocess. In this research, intermediates of sugar beet processing were studied as a substrate for ethanol production in order to reduce ethanol price as a biofuel. For ethanol production from the raw sugar beet juice in the stirred tank bioreactor batch and fed batch cultivation techniques were used. In horizontal rotating tubular bioreactor (HRTB) batch ethanol production from raw sugar beet cossettes was studied by different initial inoculum quantity (9.1 – 23.1 v/m). In both cases, *Saccharomyces cerevisiae* was used as a production microorganism. During batch ethanol production from the raw sugar beet juice, bioprocess efficiency was 59.48 % and in the fed batch process 72.49 %, respectively. At the same time, ethanol production in the HRTB from the raw sugar beet cossettes with inoculum of 16.7 % v/m (raw sugar beet cossettes) resulted in the highest bioprocess efficiency of 79.92 %. On the basis of experimental data it is clear that both intermediates of sugar beet processing can be successfully used for ethanol production.

Key words:

bioethanol, integrated bioprocess, raw sugar beet juice, raw sugar beet cossettes, HRTB.

Introduction

Pollution concerns and environmental issues caused by fossil fuels utilization have led to increased interest for biofuels usage. Also, increasing demands and source depletion of fossil fuels result in volatile and unstable prices which puts even greater emphasis on finding sustainable energy source alternatives. Biofuels are considered all fuels derived from renewable resources among which

* Corresponding author: bsantek@pbf.hr

the most used are bioethanol, biodiesel and biogas. Bioethanol as biofuel is mostly used as an addition to gasoline fuels where its content may be as low as 5 % or as high as 85 % (E–5, E–85). Lower ethanol content in these fuels requires no additional engine modifications while for higher ethanol content flexible fuel engines have to be used (*1-5*). Bioethanol is also used in chemical and pharmaceutical industry (green solvent). The main problem with bioethanol is that its production is still more expensive than gasoline production from fossil resuorces. However, development of integrated bioprocesses, where all operations involving ethanol production are combined into one single unit, results in significant energy saving and cost-effectivness (*5-7)*. Raw materials used for bioethanol production, can be divided into three main categories (depending on carbon source); sucrose containing, starch containing and lignocellulosic materials. Brasil and USA are the world largest bioethanol producers at the moment where Brasil has its production based on sugar cane and USA uses mostly starch containing crops like corn and grains. In Europe sugar beet represents raw material used primarily for sugar production *(8,9)*. However, sucrose from sugar beet can also represent a potential carbon source for bioethanol production. So far several investigations were conducted using intermediate of sugar beet processing, raw sugar beet juice, but little or no investigation was done using raw sugar beet cossettes.

Materials and methods

Microorganism, media and inoculum preparation

In all experiments yeast *Saccharomyces cerevisiae* was used as working microorganism. It was maintained on malt extract agar, which was also used for CFU determination. In this research two types of intermediates of sugar beet processing were used. Raw sugar beet juice with approximately 15 % of sucrose was used for ethanol production in the stirred tank bioreactor (Chemap AG, GF 0007, Switzerland). However, for ethanol production in the horizontal rotating tubular bioreactor (HRTB) raw sugar beet cossettes (approx. 14-16 % sucrose) were used. For inoculum preparation, in all experiments, raw sugar beet juice was used. All flasks for inoculum preparation were sterilized prior to inoculation and the amount of biomass necessary for ethanol production was obtained by yeast cultivation on a rotary shaker at 100 min^{-1} and 28 °C for 72 h. As additional yeast nitrogen and phosphorous source during inoculum preparation and fermentation, 1g/L of $NH_4H_2PO_4$ (p.a., Sigma-Aldrich, Germany) was added.

Ethanol production from raw sugar beet juice in stirred tank bioreactor

Stirred tank bioreactor was inoculated with 10 % v/v of yeast suspension previously grown on a rotary shaker. Working volume of bioreactor was 5 L. The bioreactor with medium (raw sugar beet juice with 1 g/L $NH_4H_2PO_4$) was sterilized at 121 °C for 20 minutes prior to inoculation. In this part of investigation two different cultivation techniques were used: batch and fed batch. Fermentations were conducted at 28 °C and pH value of the medium was kept between 4.5-5.0 by the addition of 0.1 M NaOH and 0.1 M H_2SO_4. During the first couple of hours in both experiments aeration was conducted in order to enhance biomass growth. pO_2 level was maintained at approximately 30 % of air saturation by alteration of stirrer speed and air flow rate. Feeding in fed batch process started when carbon source was almost depleted from broth. It was performed by five times addition of 200 mL of concentrated sugar beet juice (containing approx. 800 g/L of sugar).

Ethanol production from raw sugar beet cossettes in horizontal rotating tubular bioreactor

Ethanol production using raw sugar beet cossettes was conducted in the HRTB. HRTB is constructed as 0.6 m long and 0.25 m wide stainless steel tube. Prior to inoculation and raw sugar beet cosettes addition the bioreactor was sterilized at 121 °C for 30 min. Also, 1g/kg of sugar beet cossettes of $NH_4H_2PO_4$ was added. Five different experiments were conducted using different inoculum volumes in order to determine minimal water content of broth necessary for normal bioprocess conduction. Inoculum volumes varied from 9.1-23.1 % v/m of raw sugar beet cossettes. All experiments using semi-solid substrate were conducted at room temperature without pH regulation. Bioreactor was periodically rotated (4-6 times in one day) due to broth homogenization. The bioprocess was monitored by sampling the liquid and solid part (cossettes) of the broth. Cossettes were pressed to obtain liquid sample used in analytical purposes.

Analytical methods and bioprocess efficiency

Biomass growth in all experiments was monitored by centrifuging the samples for 15 minutes at 4500 min^{-1} using „Harrier 18/80, Sanyo“ centrifuge. Sediments were dried at 105 °C for 48 h and were used for biomass concentration determination. Supernatants were used for supstrate (sucrose, fructose and glucose), product (ethanol) and byproduct (acetate, lactate and glycerol) concentration determination. Acetate and lactate were only detected in experiments conducted in the HRTB due to the presence of naturally occuring heterofermentative lactic acid bacteria on sug-

ar beet cossettes. All samples were analyzed using high preformance liquid chromatography (HPLC) with Supelcogel™ C-610H column (Shimadzu CLASS-VP LC-10A_{VP}, Shimadzu, Kyoto, Japan). The alteration of cell number (CFU) concentrations during all experiments was monitored by using standard microbiological methods (Petri dishes incubated at 28 °C for 48 h). Additionally, pressed sugar beet cossettes were dried at 105 °C for dry weight content determination. Bioprocess efficiency parameters were determined by standard procedures. Ethanol conversion coefficient ($Y_{P/S}$) was calculated by the following equation:

$$Y_{P/S} = \frac{P - P_0}{S_0 - S} \qquad /1/$$

where P and P_0 are ethanol concentrations at the end and at the beginning of fermentation, respectively and S_0 and S are substrate concentrations at the beginning and at the end of bioprocess, respectively.

Bioprocess efficiency (E) was estimated as a ratio between experimental ($Y_{P/S}$) and theoretical conversion coefficient $(Y_{P/S})_T$:

$$E = \frac{Y_{P/S}}{(Y_{P/S})_T} \cdot 100\ (\%) \qquad /2/$$

where $(Y_{P/S})_T$ = 0.538 g/g is theoretical conversion coefficient of sucrose into ethanol.

Bioprocess productivity (Pr) was determined by the following equation:

$$Pr = \frac{P - P_0}{t} \qquad /3/$$

where t is cultivation time.

Results and discussion

Ethanol production from raw sugar beet juice as substrate in the stirred tank bioreactor

Preliminary results (data not shown) conducted in Erlennmayer flasks on a rotary shaker, where suitability of raw sugar beet juice as a meduim for ethanol production was tested, indicated that the addition of salt (1 g/L $NH_4H_2PO_4$) was necessary to achieve adequate conditions for yeast growth. Salt represents additional nitrogen and phosphorus source which is utilized for yeast biomass growth. After sterilization at

120 °C stirred tank bioreactor was inoculated with 10 % v/v of yeast suspension previously cultivated on a rotary shaker. At the first 12 hours of batch fermentation the medium was aerated and consequently yeast growth was observed due to the presence of oxygen, nitrogen and phosphorus sources in the broth. After that period, yeast concentration was at approximately constant level due to the fact that yeast started to produce ethanol under anaerobic conditions. As it can be seen in Fig. 1, the ethanol yield (P) in batch fermentation was 60.71 g/L and consumed sugar (ΔS) concentration 188.8 g/L, respectively. In batch fermenation following values of bioprocess efficiency parameters were observed: $Y_{P/S}$ = 0.32 g/g, E= 59.48 % and Pr = 0.42 g/(Lh).

In this research, the fed batch cultivation technique was also studied in order to increase ethanol production efficiency. Initial substrate concentration in the broth was reduced at 75 g/L to avoid substrate inhibition effect at the beginning of fermentation. During fed batch process, feeding was performed by the addition of a few portions of concentrated raw sugar beet juice (containing approx. 800 g/L of sugar). Dynamics of feeding process was controlled depending on the substrate consumption rate and the new feed portion was added to the bioreactor when substrate concentration dropped below 10 g/L. At the end of this bioprocess (Fig.1; 2), ethanol yield was

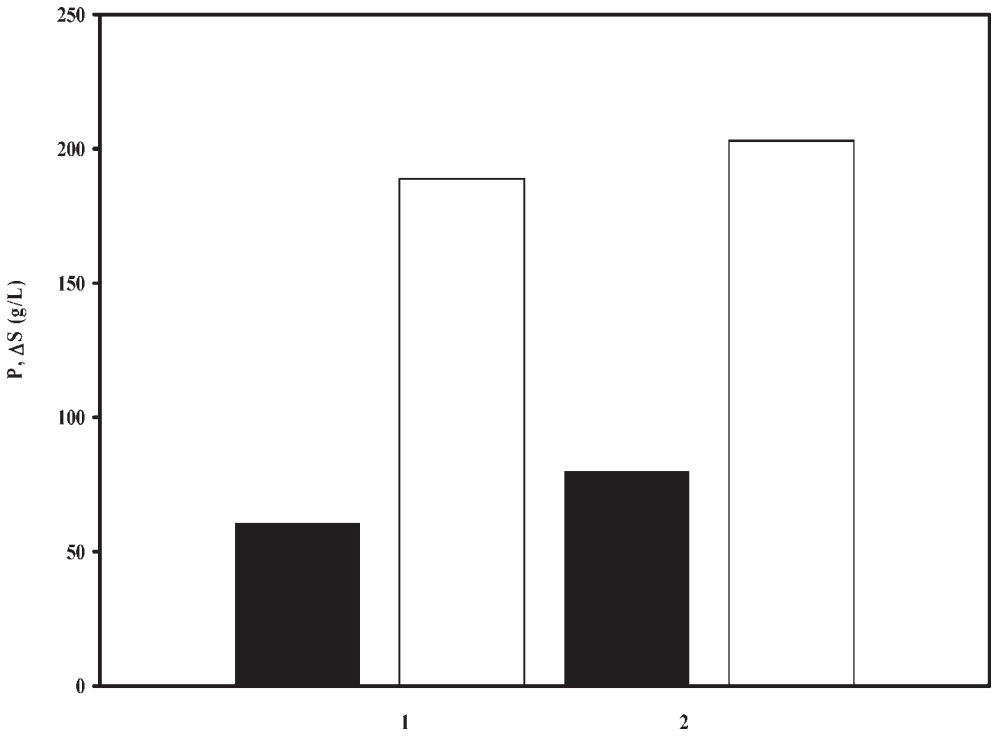


Fig. 1 – Obtained ethanol yield (P; black bars) and consumed sugar (ΔS; white bars) concentration in batch (1) and fed batch (2) fermentation of raw sugar beet juice in the stirred tank bioreactor.

79.93 g/L and consumed sugar (ΔS) concentration 203.0 g/L, respectively. Obtained results are in agreement with literature data (*10,11*). In fed batch fermentation higher values of bioprocess efficiency parameters [$Y_{P/S}$ = 0.39 g/g, E= 72.49 % and Pr = 0.42 g/(Lh)] were observed compared to the batch fermentation.

Ethanol production from raw sugar beet cossettes in the HRTB

The second part of this research was conducted in the HRTB where raw sugar beet cossettes served as a raw material for ethanol production. In this investigation, ethanol fermentation was monitored by sampling liquid as well as solid part (beet cossettes) of the broth. After taking out from HTRB, the raw sugar beet cossettes were pressed to obtain liquid samples for analysis. The main goal of this reserch was to define the minimal liquid content of the broth that is required for ethanol fermentation with yeast *S. cerevisiae*. Therefore, ethanol fermentation in the HTRB was performed with different initial volumes of inoculum (9.1-23.1 % v/m of raw sugar beet cossettes) at room temperature (20±2 °C). During these fermentations, HRTB was periodically rotated (5-6 times / day) in order to homogenize the bioreactor content. At the beginning of all experiments in the HRTB, dry matter content of raw sugar beet cossettes was approximately 23 % and at the end of fermentation 12 %, respectively. In all experiments, it was observed that sugar concentration in the liquid part was slightly lower than in the solid part of the broth due to the fact that certain time (approx. 20 h) is required for sugar diffusion from the cossettes into the liquid. Furthermore, at the start of all HRTB experiments ethanol concentration was approximately 15 g/L as a consequence of ethanol addition with yeast suspension. Initial yeast cell number in the liquid part of the broth was approx. 10^8 CFU/mL. In all experiments, the slightly increase of dry biomass and cell number were observed due to the biomass growth. Some oscillations in the dry biomass were detected as a consequence of experimental errors and system heterogeneity. In all experiments, temperature was kept at approximately constant level during the whole investigation time, which indicates that the fermentation process was carried out at approximately constant rate. Results of these fermentations are presented in Fig. 2. In these experiments, consumed sugar (ΔS) range was at approximately similar level (113.38 – 119.83 g/L) with exception of the fermentation with 13 % v/m inoculum where the highest sugar consumption was observed (132.06 g/L). This phenomenon can be explained by the fact that natural contaminants (heterotrophic lactic acid bacteria; HLAB) can also use sugar present in raw sugar beet cossettes and consequently reduced amount of sugar could be used for ethanol production. In these experiments, HLAB cell number varied from initial 10^4 CFU/mL to final 10^7 CFU/mL. The highest ethanol concentration (48.58 g/L) was observed by the fermentation with inoculum of 16.7 % v/m (raw sugar beet cossettes) what clearly shows that this inoculum quantity is sufficient for successful ethanol fermentation in the HRTB. On the basis of these results, it is clear that non-sterile raw sugar beet cossettes can be directly used for ethanol fermentation.

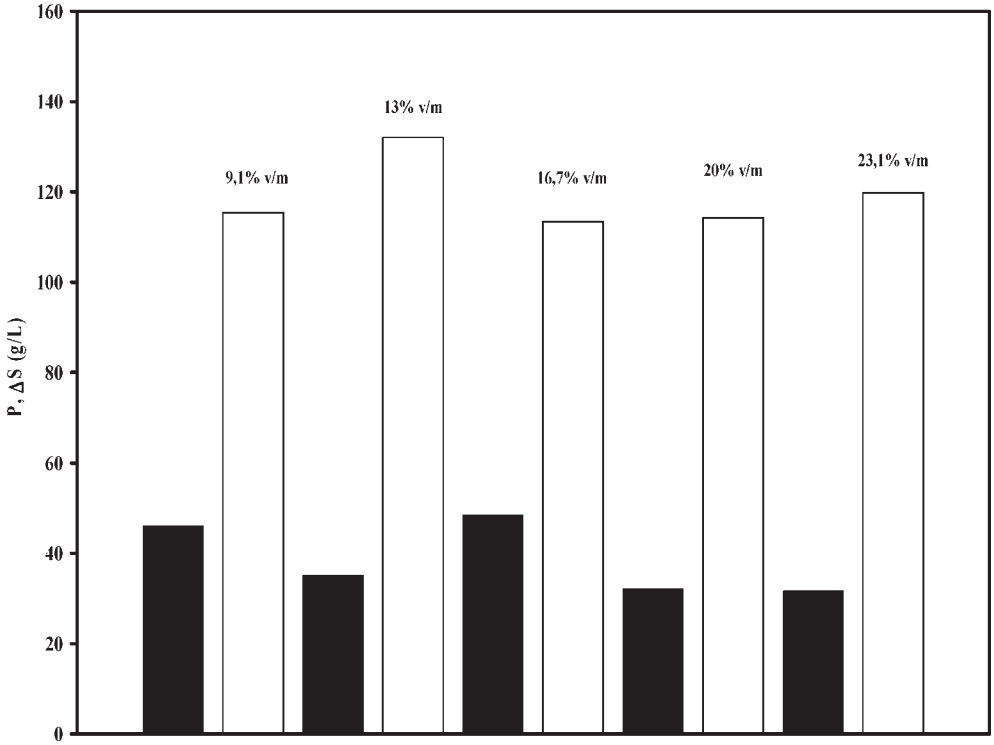


Fig. 2 – Obtained ethanol yield (P; black bars) and consumed sugar concentration (ΔS; white bars) in batch fermentations of raw sugar beet cossettes by different initial inoculum quantity (9.1 – 23.1 v/m) in the HRTB.

Comparison between these experiments was also done on the basis of bioprocess efficiency parameters. For theoretical conversion coefficient, sucrose conversion coefficient was chosen due to the fact that sucrose is a prime sugar (>95 % of total sugar content) in the sugar beet juice (*11*). As it can be seen in Table 1, conversion coefficient ($Y_{P/S}$) was in the range of 0.26 – 0.43 g/g, bioprocess efficiency in the range of 49.30 – 79.92 % and productivity in the range of 0.187 – 0.476 g/(Lh), respectively. It is clear from the obtained results that the highest values of bioprocess efficiency parameters were observed by the fermentation with inoculum of 16.7 % v/m (raw sugar beet cossettes) in the HRTB. Further increase of inoculum quantity in the HRTB is also related to the increase of free water content in the broth, which consequently has an impact on the energy demand for ethanol separation by distillation. Comparison between ethanol production from raw sugar beet cossettes and juice shows similar bioprocess behavior and efficiency. This result clearly shows that raw sugar beet cossettes can be successfully used for ethanol production, which could considerably simplify ethanol production from the energetic and economic point of view.

Table 1 – Bioprocess efficiency parameters obtained during ethanol production from raw sugar beet cossettes in the HRTB.

Inoculum quantity (% v/m of RSBC)	t (h)	$Y_{P/S}$ (g/g)	E (%)	Pr (g/L · h)
9.1	93	0.40	74.30	0.496
13.0	188	0.27	49.63	0.187
16.7	102	0.43	79.92	0.476
20.0	90	0.28	52.04	0.357
23.1	68	0.26	49.30	0.467

RSBC – raw sugar beet cossettes

Conclusions

On the basis of the obtained results, it is clear that both intermediates of sugar beet processing can be successfully used for ethanol production. Raw sugar beet juice after the addition of nitrogen and phosphorus sources is a suitable complex medium for ethanol production. The use of appropriate cultivation techniques (e.g. fed batch) can significantly increase ethanol yield and consequently improve the performance and economics of the bioprocess. The use of raw sugar beet cossettes in ethanol production eliminates extraction process of sugar beet cossettes by hot water, which considerably reduces energy demand for ethanol production. At the same time, this fact also has a considerable impact on the final price of ethanol as a biofuel. Furthermore, it is necessary to point out that further research of ethanol production from the raw sugar beet cossettes is required in order to define in more details bioprocess behavior in the HRTB.

Acknowledgement

The authors are grateful for financial support provided by the Ministry of Science, Education and Sports of the Republic of Croatia (Grant No. 058-0581990-2004).

References

1. M. Enguídanos, A. Soria, B. Kavalov, P. Jensen, Techno-economic analysis of bio-alcohol production in the EU: a short summary for decision-makers, *Report EUR 20280 EN*, (2002) (*http://www.senternovem.nl/mmfiles/26432_tcm24-279847.pdf*).

2. J.M. Henke, G. Klepper, N. Schmitz, Tax exemption for biofuels in Germany: Is bio-ethanol really an option for climate policy?, *Energy 30* (2005) 2617-2635.

3. A.K. Chandel, E.S. Chan, R. Rudravaram, M. Olakshmi Narasu, L. Verkateswar Rao., P. Ravindra, Economics and environmental impact of bioethanol production technologies: An appraisal, *Biotechnol. Mol. Biol. Rev. 2* (2007) 14-32.

4. 2010 biofuel target within reach. In: *F.O. Licht's World Ethanol and Biofuels Report*, Vol. 4, Tunderbridge Wells, UK (2006) (*http://www.agra-net.com/portal2/home.jsp?template=showissue&pubid=ag072&seqnum=416*).

5. C.A. Cárdona, Ó.J. Sanchez, Fuel ethanol production: Process design trends and integration opportunities, *Bioresour. Technol. 98* (2007) 2415-2457.

6. M. Pfeffer, W. Wukovits, G. Beckmann, A. Friedl, Analysis and decrease of the energy demand of bioethanol-production by process integration, *Appl. Therm. Eng. 27* (2007) 2657-2664.

7. M. Balat, H. Balat, H. C. Öz. Cahide, Progress in bioethanol processing, *Prog. Energy Comb. Sci. 34* (2008) 551-573.

8. C. Berg, World fuel ethanol: Analysis and outlook (2004) (*http://www.distill.com/World-Fuel-Ethanol-A&O-2004.html*).

9. E. van Thuijl, E.P. Deurwaarder, *European Biofuels Policies in Retrospect*. Energy Research Centre of the Netherlands (ECN), Petten, the Netherlands (2006).

10. S. Henke, Z. Bubník, A. Hinková, V. Pour, Model of a sugar factory with bioethanol production in program Sugars™, *J. Food Eng. 77* (2006) 416-420.

11. J.C. Ogbonna, H. Mashima, H. Tanaka, Scale up of fuel ethanol production from sugar beet juice using loofa sponge immobilized bioreactor, *Bioresour. Technol. 76* (2001) 1-8.

Amylolytic Activity Inhibition of Low Falling Number Flours by Pulses Extracts and Impact on Final Bread Quality

Boris Kovač* *Preliminary Communication*

R&D department, Food industry Mlinotest d.d., 5270 Ajdovščina, Slovenia

Yeast-leavened and sourdough breads represent one of the oldest biotechnical applications. A modern baking process may take advantage of biotechnology in its widest sense, from the improvement of cereal grains and starter cultures by recombinant DNA technology, through the use of enzymes or enzymes inhibitors as processing aids. The influence of supplementing pulses extract, containing natural amylase inhibitors on amylolytic activity of wheat flour with low falling number was studied. The amylase activity in flour was indirectly observed with the falling number (FN) method. The research shows a positive correlation between pulses flour supplement and the rise of FN of wheat flour. The result of adding bean flour is a smaller volume of the bread. The organoleptic characteristics of the bread do not change, but its freshness is improved. Correlations between different concentrations of extract and rheological properties of dough were observed by farinograph.

Key words:
amylase inhibitors, bean, falling number, farinograph

Introduction

Optimal quality of the ingredients is crucial for the final properties of baked products. Flour quality plays the main importance. Grain kernels may start to germinate, or sprout if they are exposed to long periods of rain during the final weeks before harvest. Germination begins when kernels absorb water and generate enzymes that break down stored starch and protein in the endosperm. The enzymes release sugars from starch and amino acids from proteins which nourish the growing embryo. Rheological characteristics of such flours can drastically worsen, because of the enzymatic activity, in particular beta-amylase, which causes degradation of the starch macromolecules that are important for baking. The potential contamination and growth of fungi in the rainy season may also affect the activity of amylase (1).

*Corresponding author: boris.kovac1@siol.net

Alpha-amylase is one of the enzymes produced in the sprouting kernel. Although some alpha-amylase enzyme is present in the embryo or germ of sound wheat kernels, when germination begins the embryo and layers surrounding the starchy endosperm produce the enzyme at an accelerating rate. Wheat containing very low levels of severely sprouted kernels may exhibit significant amylase activity. Alpha-amylase converts starch into sugars in the sprouting kernel, and similarly breaks down the starch granules in wheat flour when mixed with water to make bread dough. Alpha-amylase activity affects bread quality. Flour with a low Falling Number will produce bread with poor texture, a sticky bread crumb and will be difficult to process. Bread made from flour with a high Falling Number can result in loaves with poor volume and a dry bread crumb. The shelf life of the bread will also be adversely affected. Therefore it is important to use flour with the correct Falling Number (FN).

However, the milling industry is facing problems, especially in poor harvest seasons, how to use flours with high Falling Numbers but still acceptable rheological properties. A massive economic loss can be caused on a global scale because the down-graded flours have to be used for non-food purposes. Defective flours with a low Falling Number can be used in baking industry if the gluten has appropriate quantitative and qualitative values (2). Flours with a high Falling Number should be used in baking if we managed to reduce the activity of amylase with inhibitors. However, there is no available data of how to use flours with low Falling Numbers. Down-graded grain and grain products should be used in the animal feed industry.

It is well known that enzymes should be inhibited by inhibitors present in various plant materials. Amylase inhibitors are relatively thermostable proteins which have an inhibitory effect on the amylase of the pancreas and can be found naturally in aqueous pulses extracts and in some cereals. An inhibitory activity can also be found in prepared foods due to inhibitor's high thermostability.

Marshall and Lauda (3) found that kidney beans, *Phaseolus vulgaris*, contain a proteinaceous inhibitor of alpha-amylase, named phaseolamin. Legume seeds and its flours contain amylase inhibitors (4), which affect the reduced activity of the latter. There are many reports about the application of amylase inhibitors. Some amylase inhibitors become active only after a prior incubation with the enzyme. According to Harry (5), α-amylase inhibitors can be used to inhibit the amylases of saliva and pancreas in order to reduce the starch degradation.

Griffith (6) demonstrated that grape seed extract shows α-amylase inhibitory activity to acarbose; and white and green tea extracts showed a weaker α-amylase inhibition. In α-glucosidase experiments, grape seed, green and white tea extracts inhibited enzymatic activity much stronger than acarbose.

Amylase inhibitors of plant origin should be preferred in baking industry, because we do not want to incorporate any chemical substances into the products. The aim of the research is to determine the effect of the pulses flour supplement on the change of FN value in flours with different FN values in order to improve their rheological characteristics. In our previous study (7), five varieties of beans have been tested, the significant effect on the FN change was observed with Top Crop bean. Rheological characteristics and effect on dough preparation have not been tested. Varieties Top Crop and Bergold have also been included in this study. Soya beans, broad beans, peas and lentils have been tested in this research along with two kinds of beans, kidney beans and Top Crop beans. Further research will examine the effect of the pulses supplements on the rheological characteristics of the test and the quality of the bread which was prepared from flour with a lower FN than optimal.

Materials and Methods

FN determination

FN was determined by AACC Method No. 56-81.03, **(**8) by Perten Instruments. Principe: The Falling Number Method uses the starch contained in the sample as a substrate. It is based on the rapid gelatinization of a suspension of flour or meal in a boiling waterbath and the subsequent measurement of the liquefaction of the starch by alpha-amylase. The FN is defined as the total time in seconds from the start of the instrument until the stirrer has fallen a measured distance is registered by the instrument.

Brabender Farinograph Method

Rheological characteristics were determined by AACC International. Approved Methods of Analysis, Method 54-21.02 (9) by the instrument Brabender Farinograph. Principe: Standard specifies a method, using the Brabender Farinograph, for the determination of the water absorption of flours and the mixing behaviour of doughs made from them. The method is applicable to flour and meal from wheat. The resistance of the dough against the blades, which depends on the viscosity of the dough, causes an opposite deflection of the motor housing. This deflection is measured as torque and recorded and plotted on-line as a function of time in a diagram and numeric data.

Preparation of the bean flour and the bean flour extract

Beans were ground in Perten Laboratory Mill LM 3100. The extract was prepared from the 25% suspension of flour in phosphate buffer pH 6 (prepared from: K_2HPO_4 2,0g, KH_2PO_4 8g, water 1000 ml) and was filtrated through a paper filter. The following pulses were used: common bean, var. Top Crop, Semenarna, Ljubljana, lot 0608/150-OC, 15. 10. 2012; common bean, var. Bergold, Semenarna Ljubljana, lot D:02298/112-OC, 15. 10. 2012; soya bean, Biotop zelena soja, lot 16. 7. 2012; broad bean, Semenarna, Ljubljana, lot 0609, 15. 10. 2012; pea, Agrina, lot 1230, 12. 3. 2013; lentil, Biotop, lot. 15. 4. 2013.

Dough preparation

Dough was prepared using wheat flour T-500, manufacturer Mlinotest lot best before 25. 8. 2014; 2% fresh yeast Fala, lot used by 25.10.2012, 1.9% salt, bean flour and water. The dough included bean flour and not its extract. The amount of bean flour was equivalent to addition of 0%, 4%, 8% and 12% of 25% of the aqueous extract. A DK mixer was used to knead the dough. After a 10 minutes resting period, the dough was weighed to 600g, hand molded, panned and proofed for 60 minutes in a dough proofer with temperature at 35°C and a relative humidity of 90%, manufacturer Miwe, type Miwe aero. After 60 minutes, the surface of the dough was sprayed with water and the dough was put in the oven, manufacturer Miwe, type Miwe aero. Bread was baked at 220°C for 35 minutes.

Bread volume measurement

Bread volume was measured using a seed displacement method. Bread was placed into a container with a known volume which was filled with sesame seeds. The difference between the known volume of the container and the volume of the added sesame seeds represents the bread volume.

Results and Discussion

The Falling Number (FN) Method, which is commonly used in the milling and baking industry, was used to evaluate the amylolytic process. The influence of time on the extraction of possible inhibitors in the aqueous extract was tested in the first experiment. Bean flour of kidney beans was used in this experiment. The extract which was obtained by the different times of the extraction was added to the flour with high am-

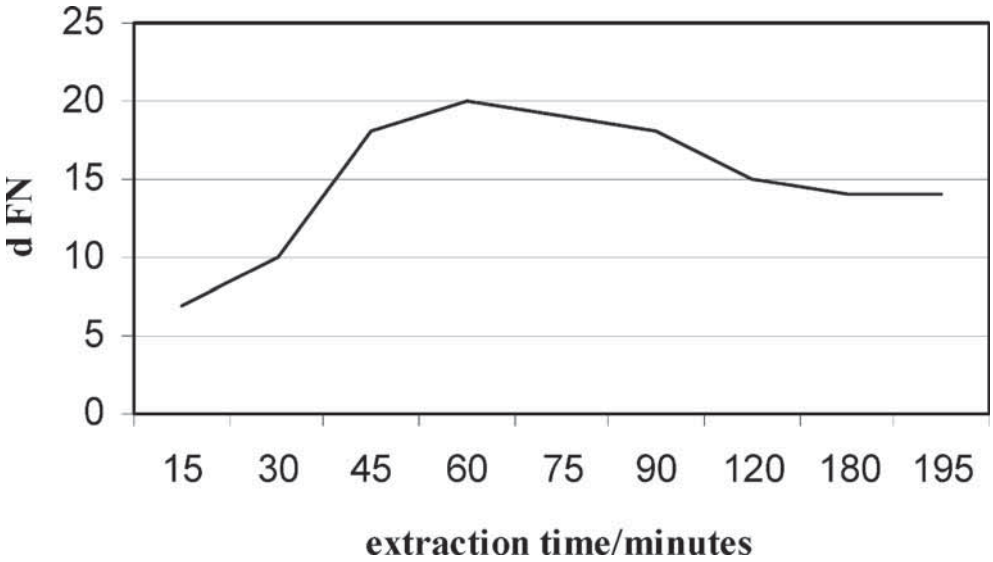


Fig. 1 – Correlation between extraction time and FN change (dFN)

ylolytic activity, of FN 190, i.e. sub-optimal Falling Number, after filtration. Results are shown in Fig. 1. The results indicate that the optimal extraction time is 60 minutes.

After 60 minutes, FN did not drastically change, but it but slightly decreased. Based on these results, we decided that the preferred time for extraction is 60 minutes. Such filtration is easier, because the majority of the parts already settles to the bottom of the flask and does not interfere with the filtration.

The potential of the reduction of FN of different pulses flours has also been tested. A 25% aqueous extract was prepared from a flour of six pulses with a 60-minute extraction. An aqueous extract was deliberately used instead of a phosphate buffer, because the distilled water is used as a carrier medium for determining the FN. Flours with a low FN value of 180, 210, 230 and flour with a high value of 350 FN were added to aqueous extracts of various pulses flours. The volume of water estimated for the suspension from the basic FN method was reduced by the amount of the added extract. The results of the analysis are presented in Table 1.

Results from previous researches have shown that the concentration of the inhibitors is higher in the samples of mature legume seeds as it is in the immature seeds. Mature legume seeds were therefore used in this research. The results show that bean flours contribute to the largest increase in FN. The effect is significant in all three initial FN values. FN values are increased regardless the initial FN value of the flour. The fact that the FN increases indicates an inhibitory character of the extract used. The greatest effect was achieved with bean varieties Top Crop and Bergold. The effect of lentil,

Table 1 – The average change in FN samples of flour with different initial FN values because of the 10% pulses flour extract supplement.

Name / Latin name	ΔFN initial FN 180	ΔFN initial FN 210	ΔFN initial FN 230	ΔFN initial FN 350
Soya bean / *Glycine max*	10	15	12	8
Broad bean / *Vicia faba*	19	16	18	18
Pea / *Pisum sativum*	5	5	4	6
Lentil / *Lens culinaris*	13	15	10	13
common bean, var. Top Crop / *Phaseolus vulgaris L. var. vulgaris*	37	38	41	40
common bean, var. Bergold/ *Phaseolus vulgaris L. var. vulgaris*	36	35	38	38

soya bean, pea and broad bean extract was significant, but lower if compared to common beans. Furthermore, bean Top Crop was also included in the study of the rheological properties of the dough. The supplement of Top Crop bean flour extract increases the FN value from the initial 180 to 217, which is 37 units; from 210 to 248 units, i.e. 38 units; and from 350 to 390 units, i.e. 40 units. Optimal bread can not be baked from tested flours with low FN values of 180 and 210, because the amylolytic activity of such flours is too high and the bread crumb is usually too sticky. FN value of about 250 units enables, if the other quality parameters of flour are appropriate, optimal properties of the final product – bread. The results indicate that 10% supplement of bean flour extract can stop the negative properties of flours which have slightly lower FN than optimal and improve their rheological characteristics.

Table 2 – Farinograph rheological characteristics and FN in correlation with bean var. Top Crop flour extract supplement.

	% of extract supplement			
	0	4	8	12
Water absorption/%	58,9	59,6	60,8	61,9
Development time/minutes	3,5	6,7	6,6	6,2
Stability/minutes	11,0	10,8	8,6	7,1
Degree of softening / farinograph units	17	23	24	33
Degree of softening (ICC) / farinograph units	43	61	70	88
Farinograph quality number / farinograph units	116	120	119	100
Falling Number/units	250	258	260	265

A farinograph analysis was performed in order to assess the influence of the added supplement on the rheological properties of the dough. The results are shown in Table 2. The farinograph analysis shows that the supplement deteriorates the rheological characteristics of the flour. However, the supplement has a positive effect on the absorption of water which increases proportionally with the increasing concentrations of the bean flour supplement. Previous research (10) shows that bean flour has higher water absorption capacity as wheat flour. The logical consequence of the bean supplement is an increase in the absorption capacity. Although beans have more protein than wheat flour, this are not a gluten proteins and therefore do not contribute to the rheological characteristics of the dough. The concentration of gluten proteins, which is important for rheological characteristics of the dough, was reduced by increasing concentrations of the bean flour supplement. Therefore all rheological characteristics deteriorated. The development time of the dough also increases with the increasing of the supplement, reaching the highest at the 4% supplement and then slightly decreases. The stability of the dough slightly decreases at the 4% of the supplement, but by increasing the percent of the supplement it drastically reduces, which indicates poor rheological characteristics of the dough. The degree of softening of the dough (ICC) is similar; after 12 minutes it is dramatically increasing which predicts poor rheological characteristics of the dough. The analysis of the FN shows the FN is increasing almost proportionately with the increasing amount of bean flour in bread. It is therefore appropriate to mix bean flour with flour which has a lower FN.

Table 3 – Volume and freshness of bread in correlation with bean var. Top Crop flour extract supplement.

	% of extract supplement.			
	0	4	8	12
Volume / dm^3	1,30	1,22	1,18	1,10
Freshness / after 24 hours	4	4	5	5
Freshness / after 48 hours	3	3	4	5
Taste / after 24 hours	5	5	5	5

The organoleptic properties of bread have been assessed by a team of tasters who chose bread with a 12% extract of bean flour as the best product. The results are shown in Table 3. The testers assessed the freshness and taste of bread with 1 to 5 points. By increasing the bean flour extract the bread stays fresher. Bread which contains more bean flour is also less crumbling. The tested bread might also be negatively assessed because of a possible aftertaste due to the addition of the bean extract, but the testers evaluated all samples as excellent. However, the volume of the bread is reduced by adding the bean extract, since the relative amount of gluten,

which is an important factor in the rising and baking bread, also decreases. The reduction of the volume is correlated with the amount of bean flour supplement. Different explanations are possible for a lower bread volume. More added water can also result in the reduction of the volume. One of the possible explanations is that the bean flour components may also negatively affect the yeast and inhibit its function. This fact should be considered in further studies, while in the bakery industry, this problem can be solved with a slightly increased amount of yeast when preparing the dough.

Conclusion

The results show that pulses extracts have an inhibitory influence on amylolytic activity and raise the FN of flour with sub-optimal values. At the same time, the increase of the bean supplement deteriorates rheological characteristics of the flour which were assessed by a farinograph analysis. The greatest effect shows common bean Top Crop. The results of the testing of the final products show that a positive correlation exists between the amount of bean flour supplement and the freshness of the final product. On the basis of the organoleptic results we can conclude that the bread has a specific pleasant taste and with a higher proportion of bean flour it also remains fresh for a long time.

References

1. B. Kovač, Kemijska tveganja izdelkov iz žit: ostanki fitofarmacevtskih sredstev in mikotoksini. In Maček, J., ed. Zbornik predavanj in referatov 9. slovenskega posvetovanja o varstvu rastlin, Nova Gorica, 4.-5. marec 2009. Ljubljana: Društvo za varstvo rastlin Slovenije, (2009) 25-30.
2. P. Giorilli, S. Lauri, Il pane. Un'arte, una tecnologia. Lucisano F. ur. Zanichelli, Milano, (2000) 251 p.
3. JJ. Marshall, CM. Lauda, Purification and properties of phaseolamin, an inhibitor of alpha-amylase, from the kidney bean, *Phaseolus vulgaris*. Biol Chem. Oct 5;250 (20). (1975) 8030-8037.
4. G. Grant, J. E. Edwards, A. Pusztai, α-Amylase inhibitor levels in seeds generally available in Europe. Journal of the Science of Food and Agriculture, 67. (1995) 235–238.
5. Harry, G. Preuss. 2009. Bean amylase inhibitor and other carbohydrate absorption blockers: effects on diabesity and general health. Journal of the American College of Nutrition, 28 (3): 266-276.
6. A. Griffith Inhibition of α-Amylase and α-Glucosidase by Bioflavonoids. Retrieved from: http://ir.library.oregonstate.edu/xmlui/bitstream/handle/1957/29953/Griffith%20Thesis%20Final.pdf?sequence=1 (2012) on September 10, 2013.
7. B. Kovač, Inhibicija amilolitične aktivnosti mok z neoptimalnim padajočim številom. Acta agriculturae Slovenica, volume 97, issue 2, (2011) 125-129.

8. AACC International Approved Methods of Analysis, 11th Ed, Method 56-81.03, Determination of Falling Number, Approved 1999. AACC International, St. Paul, MN, U.S.A (1999).

9. AACC International Approved Methods of Analysis, 11th Ed, Method 54-21.02, Rheological Behavior of Flour by Farinograph: Constant Flour Weight Procedure, Approved 1999. AACC International, St. Paul, MN, U.S.A (1999).

10. M. F. Elia, G. L. Hosfield, J. D. Kelly, M.A. Uebarsax, 1997. Genetic analysis and interrelationship between traits for cooking time, water absorption, and protein and tanin content of andean dry beans, Journal of American Society of Horticulture Science, 122(4), 512-518.

Supercritical CO_2 Extraction as an Alternative to Organic Solvents in the Production of Soybean Oil

Stela Jokić[1*], Krunoslav Aladić[2], Darko Velić[1], Đurđica Ačkar[1] and Drago Šubarić[1]

Preliminary Communication

[1]University of Josip Juraj Strossmayer in Osijek, Faculty of Food Technology Osijek, Franje Kuhaca 20, 31000 Osijek, Croatia

[2]Croatian Veterinary Institute, Veterinary Department Vinkovci, 32100 Vinkovci, Croatia

People nowadays generally expect to buy a high quality, minimally processed, „natural", additive-free food high in nutritional value. Furthermore, there is a growing interest in alternative processes that can minimize the environmental impact, decrease the toxic residues, more efficiently use the sub-products and also produce higher quality foods. The unique effects of pressure appear to be able to meet these requirements. Subjecting foods to pressures is now starting to be done by some of the world's leading food manufacturers. Organic solvents, mainly hexane, have been the preferred extraction solvent for soybean oil for a long time. Supercritical fluid technology is a viable alternative to current extraction methods. Supercritical CO_2 is an ideal solvent because it is nontoxic, nonexplosive, inflammable, cheap, readily available and easily removed from extracted products. The oil extracted from soybeans with supercritical CO_2 is of much higher quality than the hexane-extracted oil. It does not contain any phospholipids, thus eliminating the degumming step. The great advantage of the extraction of soybean oil with CO_2 compared to the conventional extraction is that the refinement stages are simplified significantly and the solvent distillation stage is completely removed. In the terms of industry supercritical CO_2 extraction still hasn't replaced hexane extraction. It may be not too long to wait before supercritical fluid extraction becomes routinely used for the production of soybean oil, thus it is very important to understand the effects of different process parameters on the yield of soybean oil as well as on the oil quality. The knowledge of these influences is not only useful for the optimization and economic evaluation of the process, but also for the ability to predict the extraction process, which is useful for scale-up as well as for the design and the optimization of industrial plant which is shown and elaborated in this paper.

Key words:

supercritical CO_2 extraction, soybean oil, scale-up

[*] Corresponding author: stela.jokic@ptfos.hr

Introduction

Soybean oil is largely obtained by screw pressing, extruding-expelling or by organic solvent extraction (mainly with hexane). The oil obtained by cold pressing is recognised as being a very high quality but a considerable amount of oil is left in the press cake. Furthermore, hexane extraction achieves almost complete recovery of the oil but the solvent which is toxic and pollutant remains in the oil (Hammond et al., 2005; Sahena et al., 2009). In recent years, there has been considerable interest by the soybean industry in alternative solvents to hexane because of increasing environmental and safety concerns. There has been much speculation about using supercritical CO_2 since this technology eliminates safety issues as CO_2 is not flammable, and the soybean oil is better quality than the oil extracted with hexane. It does not contain any phospholipids, thus eliminating the degumming step. Compared to the conventional extraction, the refinement stages are simplified significantly and the solvent distillation stage is completely removed, but in the terms of industry supercritical CO_2 extraction still hasn't replaced hexane extraction, especially because such plants include high capital costs (Friedrich and List, 1982; Friedrich and Pryde, 1984; Nodar et al., 2002; Skala et al., 2002; Hammond et al., 2005).

It may be not too long to wait before supercritical fluid extraction becomes routinely used for the production of soybean oil. Thus, it is very important to understand the effects of different process parameters on the yield of soybean oil as well as on the oil quality (Mezzomo et al., 2009; Jokić et al., 2012). Many mathematical models are presented in literature to describe the supercritical fluid extraction of oilseeds. One of the most successful models is the model proposed by Sovová, often referred to as the extended Lack's plug-flow model (Sovová, 1994). For a potential commercial process application, it is essential to test the applicability of appropriate model for supercritical fluid extraction of soybean oil, which allows us to correlate and extrapolate the experimental data.

Material and methodology

Material

Supercritical CO_2 extraction was performed on the soybean cultivar "Ika" created at the Agricultural Institute Osijek in Croatia. Moisture content was determined by oven drying to constant weight at 105 °C (AOAC, 2000) and noted as percentage (11.02 ± 0.11%). Reagent-grade *n*-hexane (J.T. Baker, Milan, Italy) was used for

laboratory Soxhlet-extraction. Commercial CO_2 (Messer, Novi Sad, Serbia) was used for laboratory supercritical extraction. The CO_2 used for pilot plant extraction was 99.5% (w/w) pure and supplied by Linde Gas Hungary Co. Ltd. (Budapest). FAME mix C14-C24 (AOCS standard 3, Restek, USA) was used. Aglycone standards of daidzein, daidzin, genistin, glycitin, glycitein, and rutin, used as an internal standard, were purchased from Sigma-Aldrich (Steinheim, Germany). Standard of genistein was supplied from Riedel de Haen® (Castle Hill, N.S.W., Australia). Genistin, genistein, and daidzein were prepared in HPLC grade methanol, and daidzin, glycitin, and glycitein in ethanol, as they varied in solubility characteristics.

Determination of particle size distribution with sieving

Material was grounded and sieved using a vertical vibratory sieve shaker (Labortechnik Gmbh, Ilmenau, Germany) for 20 min. About 200 g loading were used at each sieving. The raw material size distribution was determined using a nest of 9 sieves of aperture sizes 1.4, 0.8, 0.63, 0.5, 0.4, 0.315, 0.2, 0.1 and 0.05 mm. The mass of fragments remaining on each sieve after sieving was used to calculate the distribution of fragments, which was then normalized in respect of the total mass. For evaluation of sieve analysis results the Rosin-Rammler-Bennet (RRB) distribution was chosen (Allen, 1981). The percentage by mass of particles (R) greater than screen size (d) is given as:

$$R = 100 \exp\left[-\left(\frac{d}{d_0} \right)^n \right] \tag{1}$$

where d_0 represents the particle size corresponding to the 36.8th percentile of the cumulative probability distribution (size constant), and n controls the shape of the distribution (uniformity coefficient).

The function of the sum of sieve residue (R) was fitted to the experimental data by changing the representative particle size d_0 and the uniformity coefficient n, minimizing the sum of the mean square error using *STATISTICA 8.0* software.

Determination of the initial oil content

The initial oil content was measured by traditional laboratory Soxhlet-extraction with *n*-hexane. About 30 g of ground soybeans was extracted with about 250 mL solvent, until totally depleted. The whole process took 16 h. The measurement was done in triplicate. The average of the initial oil content for three replicates was 20.19 ± 0.72%.

Supercritical CO_2 extraction

The experiments were performed on two set of apparatus, a lab scale unit (**Fig. 1**) and a pilot plant (**Fig. 2**). The main plant parts and properties of the laboratory-scale high pressure extraction plant (HPEP, NOVA-Swiss, Effertikon, Switzerland) were: the diaphragm type compressor (with pressure range up to 1000 bar), extractor with internal volume 200 mL (P_{max} = 700 bar), separator (with internal volume 200 mL, P_{max} = 250 bar), and maximum CO_2 mass flow rate of 5.7 kg/h. The height and the diameter of the extractor basket were h_E=0.148 m, d_E=0.04 m, respectively.

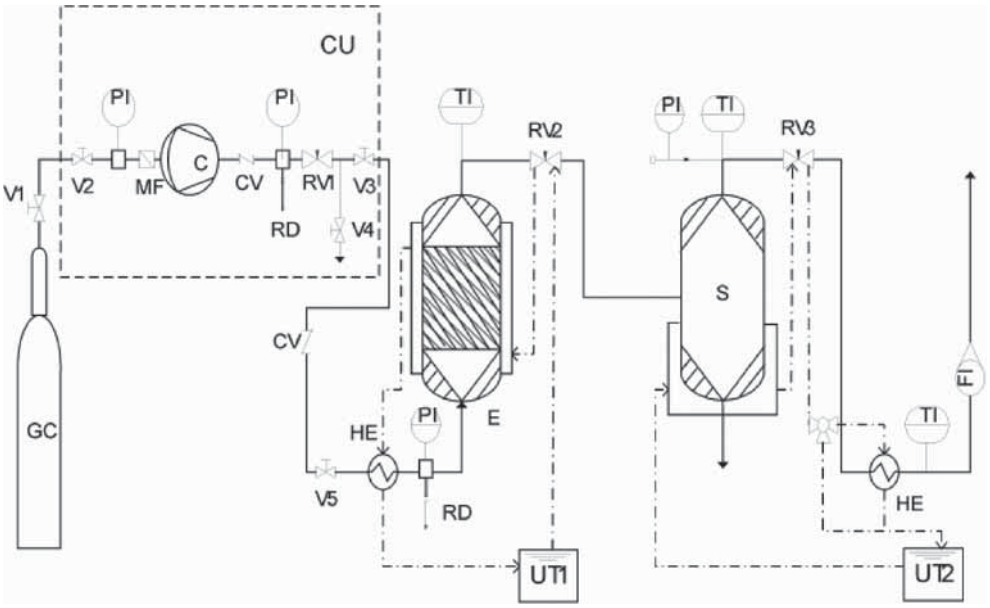


Fig. 1 – Schematic diagram of the apparatus used for supercritical fluid extraction

GC − gas cylinder, CU − compressor unit, C − compressor with diaphragm, E − extractor, S − separator, HE − heat exchanger, UT − ultra thermostat, RV – regulation valve, V − on-off valve, MF − micro filter, CV – cut-off valve, RD − rupture disc, PI – pressure indicator, TI − temperature indicator, FI − flow indicator.

The extracts were collected in previously weighed glass tubes and placed in the separator at ambient temperature and pressure. The amount of extract obtained at regular intervals of time was established by weight using a balance with a precision of ±0.0001g. Separator conditions were 15 bar and 25 °C.

Scale-up experiments

The high pressure pilot plant equipped with 5 L volume extractor vessel (delivered by NATEX Austria) was used for extraction of soybean oil. The extraction was

carried out at 400 bar and 40 °C. The operating parameters in the separator were 40 bar and 20-25 °C. The accumulated product samples were collected and weighed at certain time intervals. The CO_2 flow rate was measured by a Mikro Motion RFT 9729 type mass flow meter, and it was about 6.413 kg/h. The extraction was carried on until the amount of the product sample, collected for 1 h decreased to under 0.1% of the raw material. The height and the diameter of the extractor basket were h_E=0.585 m, d_E=0.098 m, respectively. The scale-up assays (Clavier and Perrut, 2004; Jokić et al., 2012) were performed according to two proposals and compared to small scale experiments at the same pressure and temperature conditions. The first was geometric proposal:

$$\frac{h_{\,packed\ column}}{d_{\,packed\ column}} = \text{constant} \tag{2}$$

where the ratio packed column height/packed column diameter should be maintained constant. The second proposal was selected according to the mass transfer mechanism involved on the extraction. The ratio solvent flow rate/mass of raw material should be maintained constant between small and large scales:

$$\frac{\dot{m}_f}{m_s} = \text{constant} \tag{3}$$

where the m_s and m_f are the mass of the raw material and the mass flow rate of the CO_2.

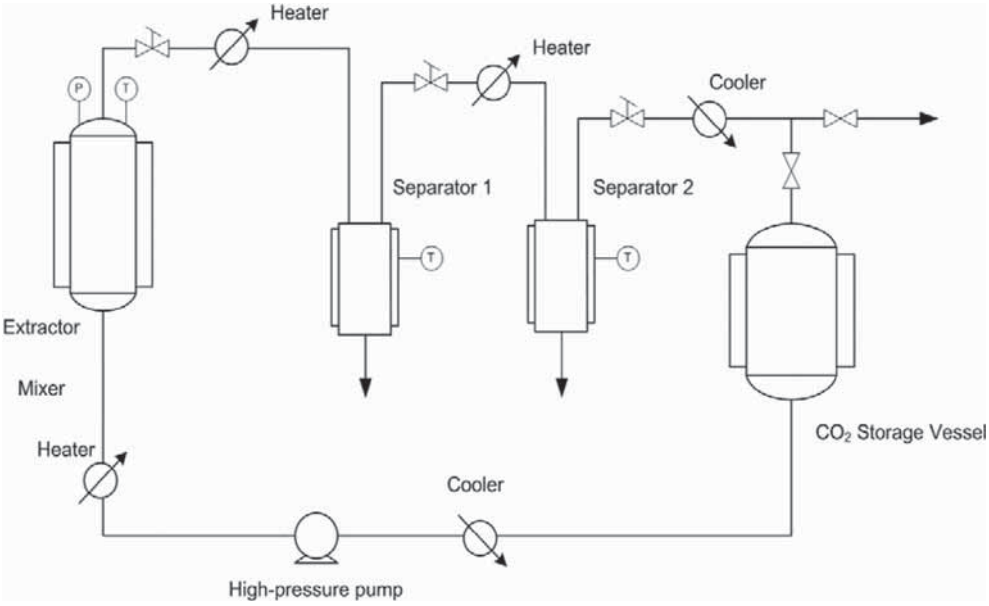


Fig. 2 – Schematic flow diagram of pilot plant extraction apparatus

Determination of fatty acid composition of soybean oil

The fatty acid composition of the soybean oil was determined by gas chromatography using an GC Agilent HP6890 N/5972, equipped with an HP 88 capillary column (100m x 0.25mm i.d.; film thickness 0.25 μm). The sample (1 μl) was injected with a split ratio of 50:1 and the inlet temperature was set at 523 K. The detector temperature was set at 553 K. The initial oven temperature was 393 K (held for 1 min), and then increased to 448 K at a rate of 283 K/min, held for 5 min, and finally increased to 530 K at 278 K/min. Helium was used as the carrier gas at a flow rate 0.8 ml/min. Total analysis time was 44.50 minutes. The FAME peaks were identified using FAME (fatty acid methyl ester) standards. The composition of the fatty acids was calculated from their peak areas.

Determination of isoflavone content in soybean meal

Soybean seed meal (10 g) was extracted by 70% ethanol (50 ml). The extraction process was carried out using ultrasonic bath (Branson and Smith-Kline Company, B-220) at the room temperature for 40 minutes. After filtration, extraction solvent was removed by rotary evaporator (Devarot, Elektromedicina, Ljubljana) under vacuum. Obtained extracts were dried at 50 °C to the constant mass. The extracts were dissolved in 2.5 mL of 80% MeOH and sonicated on an ultrasonic bath for 1h at room temperature. The solutions were filtered over 0.45 μm syringe cellulose filter, and transferred into HPLC vials. HPLC analysis of the extracts was performed using a Agilent 1200 series HPLC with RR Zorbax SB-C18 column (3.5 μm, 30 x 2.1 mm). Mobile phase A was 0.2% formic acid in water, and mobile phase B was acetonitrile. The injection volume was 1 μL, and elution at 0.45 mL/min with gradient program (0-1.24 min 2% B, 1.24-3.70 min 2-29% B, 3.70-8.00 min 29-30% B, 8.00-9.00 min 30-98% B, 9.00-10.00 min 2% B). UV detection was carried out at 260 nm. For the quantification of the isoflavones, four different concentrations of mixed standards (containing all six analyzed isoflavones) were used to make the calibration curves. Single standards were also prepared for peak identification. Isoflavone concentrations were calculated as mg of isoflavones per 1 g of extract.

Results and discussion

In our previous paper (Jokić et al., 2012) we explain in detail how different extraction parameters (pressure, temperature, CO_2 mass flow rate and characteristic particle size) influenced on the extraction yield of soybean oil. The extraction yield rate of oil from soybeans obtained by supercritical CO_2 increased with pressure related to in-

crease of seed oil solubility. The soybean oil has very small solubility at and below 300 bar. The effect of temperature on the extraction rate, at constant pressure, is due to the two mechanisms: an increase in the process temperature increases the solubility due to solute vapour pressure enhancement and reduces the solubility due to the decrease in the solvent density. The extraction rate was affected significantly by increase in solvent flow rates at the beginning of the extraction period (fast extraction period). Hence, the higher flow rate leads to a shorter extraction time to reach the same yield as the lower flow rate test. Furthermore, the decrease in particle size show higher extraction yield because of the increase in oil amount outside the particles, due to the enhancement of surface area with particle size reduction. Similar results have been published by Friedrich and List (1982) and Nodar et al. (2002).

To explore how the extractor size influences these process, the same soybean samples were extracted using equipment with different extraction volumes (0.2 L and 5 L). The Sovová's model was used to describe the extraction curves obtained by small and large scale assay (Jokić et al., 2012). **Fig. 3** show the extraction curves obtained at the lab and pilot plant equipment. The extraction ratio was plotted versus time. It can be seen excellent agreement between obtain extraction oil yield using small scale

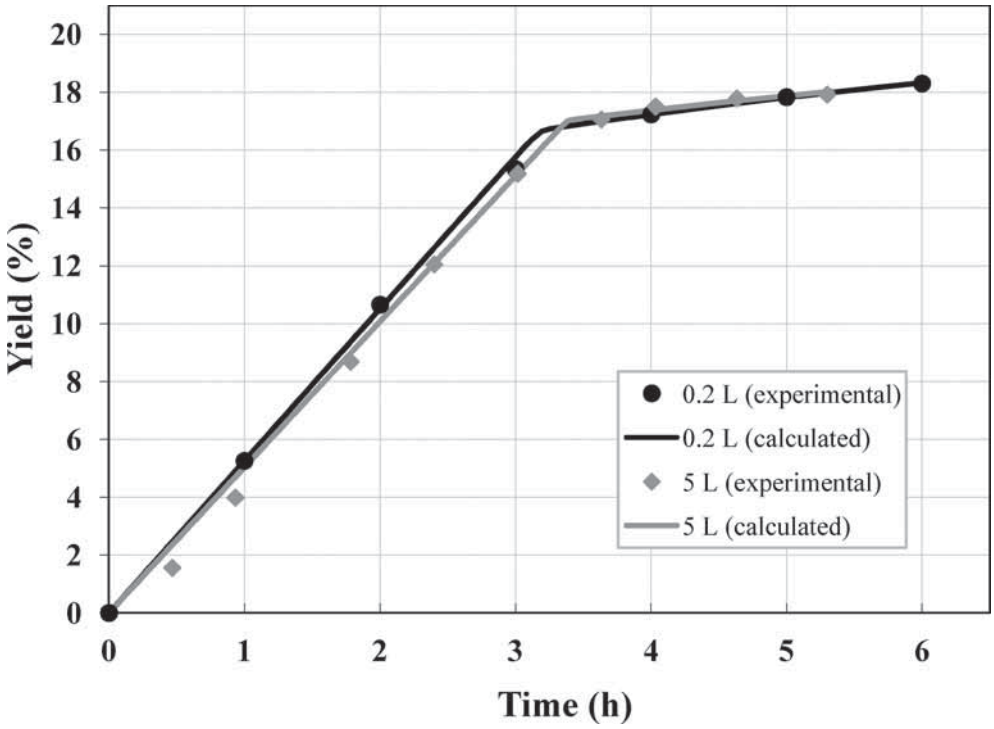


Fig. 3 – Experimental and modelled curves (Sovová'model) for small scale and scale-up proposal. Conditions: T_E = 40 °C; P_E = 400 bar; d_0 = 0.383 mm; m_f = 0.436 kg/h for small scale and m_f = 6.413 kg/h for pilot plant

compared to pilot scale which means that supercritical extraction of soybean oil could be predicted by the used scale-up proposals. Furthermore, from **Fig. 3** can be seen a good model adjustment for all curves in small and large scales. In previous studies (Mezzomo et al., 2009) the supercritical extraction of peach almond oil has been investigated and four scale-up methodologies, based on mass transfer mechanisms, were applied. The results indicate that the scale-up from laboratory to pilot plant was also successfully performed on the basis of these two assumptions.

The fatty acid composition of soybean oil extracted by supercritical CO_2 (SC-CO_2) at 300 bar and 40 °C was summarised in **Table 1**. The main fatty acids of the oil extracted with supercritical carbon dioxide were: palmitic acid, stearic acid, oleic acid, linoleic acid and linolenic acid. The results indicate that the soybean oil is rich in polyunsaturated fatty acids (PUFA). Oleic acid, belonging to monounsaturated fatty acids (MUFA), is the second most abundant in soybean seed oil. The main saturated acid in the soybean seed oil is palmitic acid, followed by stearic acid. The essential fatty acids like linoleic acid are not synthesized in the human system and must be supplied externally through the diet, and soybean oil can be a good nutritional supplement as a source of linoleic acid (Jokić et al., 2011).

Table 1 – Fatty acid composition of soybean oil

Fatty acids	% yield of extracted oil by SC-CO_2	% yield of extracted oil by Soxhlet
Linoleic acid	51.11 ± 0.09	43.21 ± 0.01
Oleic acid	23.79 ± 0.04	28.7 ± 0.05
Palmitic acid	12.92 ± 0.05	11.65 ± 0.04
Stearic acid	5.85 ± 0.13	8.36 ± 0.11
Linolenic acid	6.21 ± 0.08	5.67 ± 0.06

Soybean seed meal contains, after the extraction of oil, large amounts of phenolic compounds. The interest in extraction of phenolic compounds from the by-product arises because they are a source of a significant amount of antioxidative compounds (Head, 1998; Messina, 1999; Venter, 1999). Furthermore, soybean owes its recently acquired 'functional food' status to the presence of isoflavones (Riaz, 1999). The aim of this work was to determine the content of the isoflavones in soybean seed meal after the supercritical CO_2 extraction. Supercritical CO_2 extraction was performed at 300 bar and 40 °C. The content of total and individual isofavones was determined by high performance liquid chromatography and is given in **Table 2**. The total isoflavone content in soybean seed meal was 21.8 mg/g of extract. The most abundant isoflavone was genistin. Glycitein content in soybean seed meal was not reported because it was below limit of detection.

Table 2 – Content of isoflavones in soybean seed meal after the supercritical CO_2 extraction

Isoflavone	(mg/g extract)
Daidzein	3.8 ± 0.1
Genistein	5.9 ± 0.3
Daidzin	3.7 ± 0.1
Glycitin	1.3 ± 0.2
Genistin	7.1 ± 0.2

Conclusion

The oil extracted from soybeans with supercritical CO_2 is much higher quality than the hexane-extracted oil. It does not contain any phospholipids, thus eliminating the degumming step. The great advantage of the extraction of soybean oil with CO_2 compared to the conventional extraction is that the refinement stages are simplified significantly and the solvent distillation stage is completely removed. In this work the scale-up between laboratory-scale high pressure extraction plant and pilot plant was successfully performed according to the mass transfer mechanism involved on the extraction and geometric proposal. Soybeans processed into oil, leaving a by-product with large amount of phenolic compounds known as isoflavones. The most abundant isoflavone was genistin. The total isoflavone content in soybean seed meal was 21.8 mg/g of extract.

References

1. Allen T, *Particle size measurement*, Chapman and Hall, London, pp. 139-140, 1981.
2. AOAC, *Official Methods of Analysis*, 17th edition, no 925.40, Association of Official Analytical Chemists, Washington, 2000.
3. Clavier JY, Perrut M, *Scale-up issues for supercritical fluid processing in compliance with GMP*. U P. York i U.B. Kompella (Editors), Supercritical fluid technology for drug product development (pp. 627–631). New York: Marcel Dekker, Inc., 2004.
4. Friedrich JP, List GR, *Characterization of soybean oil extracted by supercritical carbon dioxide and hexane*, Journal of Agricultural and Food Chemistry **30** (1982), 192–193.
5. Friedrich JP, Pryde EH, *Supercritical CO_2 extraction of lipid-bearing materials and characterization of the products*, Journal of the American Oil Chemists' Society **61** (1984), 223–228.
6. Hammond EG, Johnson LA, Su C, Wang T, White PJ, *Edible oil and fat products: Edible oils*. U F. Shahidi (Editor), Bailey's Industrial Oil and Fat Products (pp. 592-614). Hoboken, New Jersey: John Wiley & Sons, Inc., 2005.

7. Head KA, *Isoflavones and other soy constituents in human health and disease*, Alternative Medicine Review **3** (1998), 433–450.

8. Jokić S, Svilović S, Zeković Z, Vidović S, Velić D, *Solubility and kinetics of soybean oil and fatty acids in supercritical CO_2*, European Journal of Lipid Science and Technology **113** (2011), 644–651.

9. Jokić S, Nagy B, Zeković Z, Vidović S, Bilić M, Velić D, Simándi B, *Effects of supercritical CO_2 extraction parameters on soybean oil yield*, Food and Bioproducts Processing **90** (2012), 693–699.

10. Messina MJ, *Legumes and soybeans grown foods: overview of their nutritional profiles and health*, American Journal of Clinical Nutrition **70** (1999), 439–450.

11. Mezzomo N, Martínez J, Ferreira SRS, *Supercritical fluid extraction of peach (Prunus persica) almond oil: Kinetics, mathematical modeling and scale-up*, The Journal of Supercritical Fluids **51** (2009), 10–16.

12. Nodar MD, Gómez AM, Martínez de la Ossa E, *Characterisation and process development of supercritical fluid extraction of soybean oil*, Food Science and Technology International **8** (2002), 337–341.

13. Riaz MN, *Soybeans as Functional Foods*, Cereal food world **44** (1999), 88-92.

14. Sahena F, Zaidul ISM, Jinap S, Karim AA, Abbas KA, Norulaini NAN, Omar AKM, *Application of supercritical CO_2 in lipid extraction – A review*, Journal of Food Engineering **95** (2009), 240–253.

15. Skala D, Žižović I, Gavrančić S, *Primena natkritične ekstrakcije u prehrambenoj industriji*, Hemijska industrija **56** (2002), 179-190.

16. Sovová H, *Rate of the vegetable oil extraction with supercritical CO_2- I. Modelling of extraction curves*, Chemical Engineering Science **49** (1994), 409-414.

17. Venter CS, *Health benefits of soy beans and soy products: a review*, Journal of Family Ecology and Consumer Sciences **27** (1999), 24-33.

Modernization of Production Plant and Processes in Zagrebačka pivovara from 1995 to 2012

Borislav Šćulac*

Zagrebačka pivovara, Zagreb, Croatia

Minireview

Zagrebačka pivovara was founded in year 1892 with initial capacity of 30.000 hl per year and started with production of Ožujsko beer which is nowadays the most sold Croatian beer. It was independent until 1994 when it becomes a member of Interbrew. Today is a member of MolsonCoors. A large modernization of the brewery happened during sixties and seventies following the growth of beer sales, but in nineties the equipment mostly became obsolete. In period from 1995 till 2012 altogether 63.845.000 € was invested in replacement of existing and purchasing of new equipment for beer production but also in environment protection and safety at work. Parallel with new equipment, modern processes for beer production were implemented. High Gravity Brewing with higher original extract content in wort and correction of extract content after filtration phase was implemented. Wort fermentation process was modernized with diacetyl measurement and follow up, as an indicator of fermentation end. Cold maturation process on temperature of –1 ºC was implemented in order to assure colloidal beer stability. Capital expenditures and implementation of modern brewing processes resulted with better process control, more reliable and more efficient production, and assurance of constant product quality. Also, they resulted with substantial increase of production capacity, so today brewery has production capacity of 2.000.000 hl/year. Zagrebačka pivovara is leader in innovations and today it produces 11 beer types. A lot of packaging innovations were implemented e.g. replacement of returnable glass bottles, introduction of branded crates for returnable glass bottles, introduction of PET bottles and one way glass bottles. Today Zagrebačka pivovara holds leading position on Croatian beer market with market share of 43% and leading brands in core, premium and value segments. Plans for future are connected with beer and flavor beer innovations and different packaging innovations, in order to maintain and win market share. Already in 2013, brewery invests 10.000.000 € in new embossed bottles and related packaging machines. Modernization of production equipment will take place in relation with evolution of production processes and aging of current equipment, with goal to assure quality and efficiency, and to assure environmentally sustainable development.

*Corresponding author: borislav.sculac@molsoncoors.com

Introduction

Zagrebačka pivovara made wide modernization of production equipment and production processes after privatization in 1994, as well as many other organizational and managerial changes. Review of modernization scope and benefits has been done and results are presented in text bellow.

History of Zagrebačka pivovara

Zagrebačka pivovara was founded in year 1892 at current location. Small beer manufacturers couldn't cover increasing beer demand in Zagreb and it was born idea to construct new brewery. Initiators were count Gustav Pongratz and baron Petar Dragutin Turković. Production buildings were designed by architect Kun Weidmann with initial capacity of 30.000 hl/year. Opening celebration was on 12 July 1893. Facility was in front of that time, for example beer garden had electric lights, first one in Zagreb town and 15 years before than city of Zagreb introduce electricity. Beer garden with electric lights was special attraction for people. Brewery launched brand Ožujsko, which is today the most popular beer in Croatia.

Beer production was organized on traditional way, until sixties and seventies of last century when was seen strong growth of beer demand. A large modernization of the brewery happened at that time following the growth of beer sales. New buildings were built together with new equipment, but in nineties the equipment mostly became obsolete.

Brewery was independent until 1994 when it becomes a member of Interbrew. Today is a member of MolsonCoors and the biggest brewery in Croatia (1).

Modernization of production equipment in period 1995-2012

After privatization in 1994 Interbrew took over the majority of shares. Privatization was followed by huge capital expenditure with ambitious goals:
– Replacement of obsolete brewing & packaging equipment
– Production capacity increase
– Key performance Indicators improvement & efficiency increase
– Quality Assurance of processes and final products
– Assurance of environment protection
– Assurance of safety on work

In period from 1995 till 2012 altogether 63.845.000 € was invested in Production. Capital expenditure per year is seen in Fig. 1.

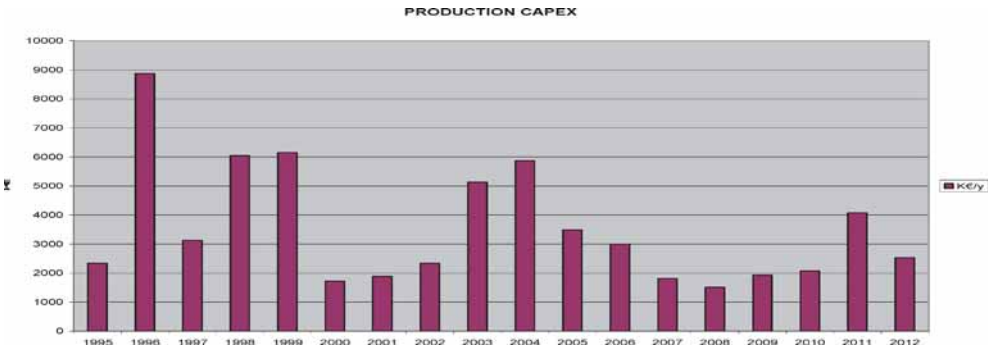


Fig. 1 – "Capital expenditure in Production per year from 1995 to 2012 (k€/year) (2)

In first years focus was replacement of obsolete equipment and capacity increase in brewhouse and fermentation areas. Later focus turned on investments related with increase of production efficiency.

The most important capital investments were:
- Brewhouse renewal: Meura Mash filter, hammer mill, wort decantation tank, wort cooler , Cleaning In Place installation and other related equipment
- New fermentation building with 17 cylindrical conical tanks, yeast propagation and yeast storage tanks, Cleaning In Place and other related equipment
- New beer filtration line with candle filter, pVpp, carbonation and blending unit
- New Bright Beer tanks
- Automation, Energy and Fluids recoveries
- Renewal and reconstruction of filling lines for returnable and one way glass
- Renewal and reconstruction of Keg line with capacity increase
- Installation of Pet filling line
- Installation of Can filling line
- Decreasing of noise emission – new facades on main packaging and brewing buildings, noise protection walls
- Renewal and reconstruction of cooling plant with increase of safety
- Utilities: new CO_2 purification plant and Water treatment upgrade
- Complete replacement of sewer system with separation of technical and rain water including new buffer basin for waste water

All in all, wide modernization of production equipment has been done in period 1995-2012.

Implementation of modern brewing processes in period 1995 – 2012

Parallel with new equipment, modern processes for beer production were implemented.

High Gravity Brewing was introduced in beer production, with wort gravity in brewhouse between 15-20 % w/w and final gravity adjustment after beer filtration phase.

Wort fermentation process was modernized with diacetyl follow up. Diacetyl reduction is set up as a measure for end of fermentation process. Diacetyl reduction is ongoing on temperature of fermentation, cooling down of green beer starts when diacetyl reduction is finished. Controlled process enables stable worth fermentation of 3-4 % w/w per day, depending on specific process parameters as temperature and yeast concentration.

Cold maturation process on temperature of -1 ºC was implemented in order to assure colloidal beer stability.

Implementation of modern brewing processes resulted with better control of brewing processes and with standard quality of finish goods.

Results of equipment and brewing processes modernization

Capital expenditures and implementation of modern brewing processes resulted with better process control, more reliable and more efficient production, and assurance of constant product quality. Also, they resulted with substantial increase of production capacity. Production capacity was increased from 1.100.000 hl in 1995 up to 2.000.000 hl/year today. Increase of production capacity enabled increase of sales volume which came afterwards, seen in Fig. 2.

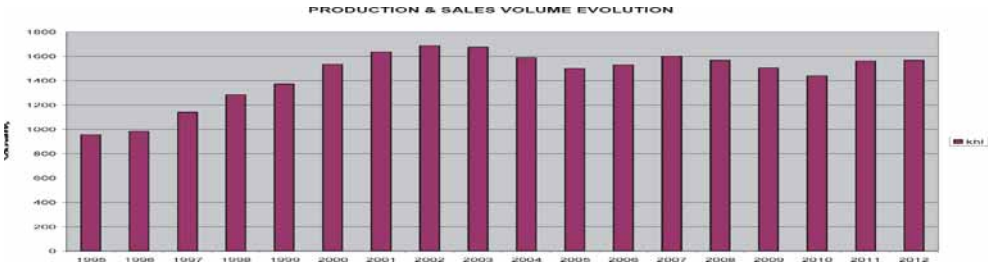


Fig. 2 – Evolution of Production & Sales beer volume from 1997 to 2012 (khl) (3)

Production Efficiency is followed by KPI (Key Performance Indicator). The most important KPI with the biggest cost impact are Total Technical Productivity, Water consumption, Heat consumption and Electricity consumption.

Total Technical Productivity is defined as ratio between beer volume produced in hl/year and number of working hours of all production employees per year.

Water consumption is defined as ratio between water spent in production in hl/year and beer volume produced in hl/year.

Steam consumption is defined as ratio between steam spent in production in MJ/year and beer volume produced in hl/year.

Electricity consumption is defined as ratio between electricity spent in production in kWh/year and beer volume produced in hl/year.

Evolution of mentioned KPI is seen in Figures bellow. Positive KPI evolution resulted in significant production cost reduction.

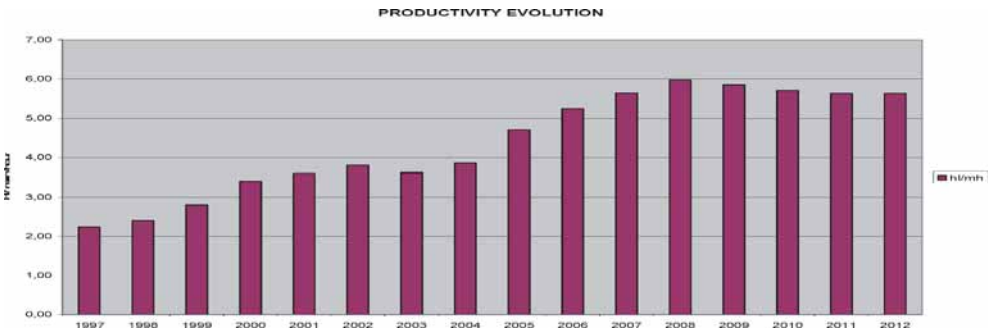


Fig. 3 – Evolution of Total Technical Productivity from 1997 to 2012 (hl beer/working hour) (3)

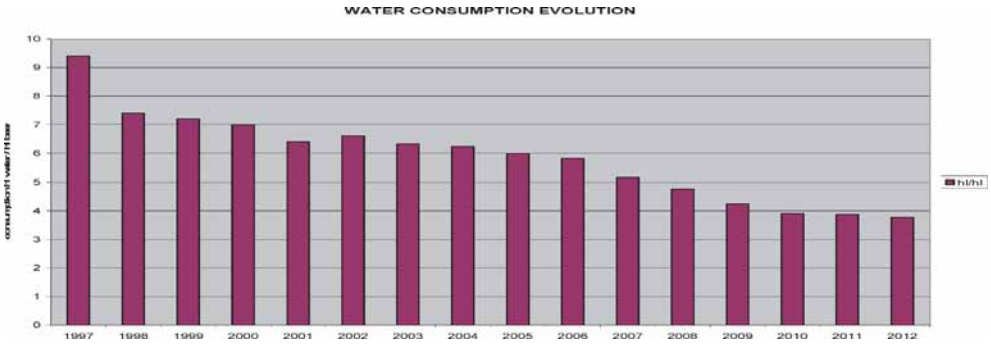


Fig. 4 – Evolution of Water Consumption from 1997 to 2012 (hl water/hl beer) (3)

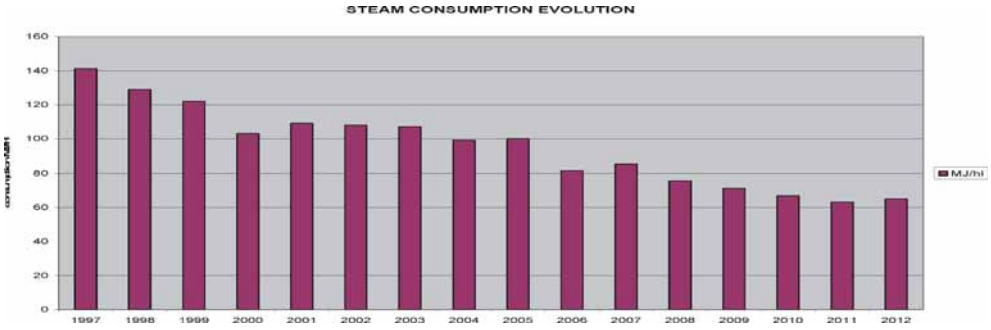


Fig. 5 – Evolution of Steam Consumption from 1997 to 2012 (MJ/hl beer) (3)

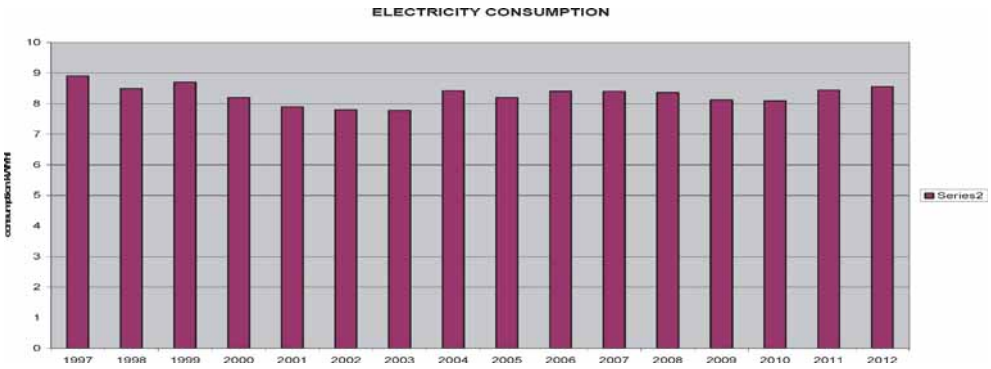


Fig. 6 – Evolution of Electricity Consumption from 1997 to 2012 (kWh/hl beer) (3)

Development so far and plans for future

So far brewery managed wide modernization of production equipment in period 1995-2012 but also put in place huge resources in fields of Human Resources, Sales and Marketing, to strengthen and develop beer brands and portfolio, supported by Management System developed for brewing industry.

Zagrebačka pivovara is leader in innovations on Croatian beer market and today it produces 11 beer and beer-mix types. It is also leader in packaging innovations as well:

– First on Croatian beer market with branded crates made of polyethylene
– First on Croatian beer market with complete replacement of old bottles
– First on Croatian beer market with PET packaging
– Complete beer portfolio – 11 beer and beer-mixes brands

All efforts resulted with market share increase from 29% in 1995 to 43% in 2013. Today Zagrebačka pivovara holds leading position on Croatian beer market, with leading brands in core, premium and value segments.

Plans for future are connected with beer and flavor beer innovations and different packaging innovations, in order to maintain and win market share further on. Already in 2013, brewery invests 10.000.000 € in new embossed bottles and related packaging machines. Modernization of production equipment will take place in relation with evolution of production processes and aging of current equipment, with goal to assure quality and efficiency, and to assure environmental sustainable development.

Conclusion

Zagrebačka pivovara managed wide modernization of production equipment in period 1995-2012 with the goals to replace obsolete equipment, increase capacity, assure product quality and assure compliance with environmental regulations. Total Production Capital Expenditure in this period was 63.845.000 □. Modern brewing processes were implemented as well. Development assured modern, efficient and sustainable operations on original location but also enabled sales volume growth. Today Zagrebačka pivovara is market leader on Croatian market with 43% market share and sales volume of 1.600.000 hl, with leading brands in all market segments. In next period brewery will continue both with further modernization of production and innovations, to assure successful and sustainable business results.

Literature

1. Archive of Zagrebačka pivovara
2. Capex reports Y 1995-2012 – Internal documents of Zagrebačka pivovara
3. Key Performance Indicators reports Y 1997-2012 – Internal documents of Zagrebačka pivovara

Overview of the Past and the Look on the Future of Forest Reproductive Material Production in Croatia

Sanja Perić, Martina Tijardović* and Tomislav Dubravac *Review*

Croatian Forest Research Institute, Division of silviculture, Jastrebarsko, Croatia

Forest reproductive material represents basis for all activities related to forest establishment (afforestation of available forest areas and abandoned agricultural land, burned areas, landfills, queries, protective belts and help to natural regeneration). Increase of negative influences on natural ecosystems together with increase of public needs for all functions which forests provide open the space for more intensive nursery production in the future. This is the reason why afforestation in the future should have more pronounced significance in the Croatian economy if we take into the account the fact that besides utilization of forest products, ecological and other services forests also provide economical benefits in the frame of signed EU agreements. Croatian Forest Research Institute was established during 1960-s with the aim of forest culture establishment with different purposes and was the leading institution in production of forest reproduction material. From the 1992 Institute continually conducts expert supervision of nursery production which points to real needs not only in artificially established forest cultures but in natural stands as well. Five year monitoring data according to nurseries, tree species and cultivation methods fulfilled with overview on production trends from the beginning of expert supervision (from 1992) highlight the negative production trend. Estimations of future production and all available areas for forest production will be harmonized with Strategy of rural development 2014-2020. Besides autochthonous tree species special overview on past and present production of pioneer species will be presented. Those species are suitable for establishment of cultures for energy purposes (e. g. willows, poplars, indigo bush). Rare and endangered tree species have special place in the scope of forest biodiversity. Guidelines for nursery production development will contribute not only to forestry but to other sectors as well.

Key words:

forest tree species, nursery production, development guidelines, Strategy.

*Corresponding author: martinat@sumins.hr

Introduction

Biotechnology by its broad definition presents the application of science and technology to living organisms, as well as parts, products and models thereof, to alter living or non-living materials for the production of knowledge, good and services (OECD official web page). Forestry has been defined as scientific management of forests for the continuous production of goods and services (e. g. Perry 1998) and presents significant segment of biotechnology. Significance of forestry increases and becomes especially highlighted with the growing changes of ecological conditions (Tijardović et al 2013) which have led to the number of natural disturbances (e. g. storm event in Spačva basin in 1998, 2008 – Dubravac and Dekanić 2009). For example, more than 130 separate wind storms have been identified as causing noticeable damage to European forests in the last 60 years (Gardiner et al 2011). Strom Lothar which in 1999 struck Europe, created largest storm damage to European forests ever recorded and resulted in destruction of some forest ecosystems with great financial damages. However, the growing negative force upon forest ecosystems derive from synergy of individual negative influences like site pollution, change in seed periodicity, increase of climatic extremes, change in pest dynamics, introduction of invasive plant species (e. g. indigo bush), unprofessionally conducted interventions, changes in soil water table, etc.

Among others, forestry encompasses activities in land use management, land use change and afforestation together with management of existing forests (protection, tending, regeneration, conversion, etc.). There are different reasons for forest establishment which include provisioning services (provision of wood and other forest products), regulating services (protection of water, soil, influence on local climate, conservation of biodiversity, etc.) and cultural services (EFI Atlantic 2013). During the history, production of wood has been highlighted in Croatian forestry (Perić 2001, Perić et al 2003, Perić et al 2004). However, in the last decades the role of forests in providing protective functions together with other forest services has been recognized. Forest reproductive material (FRM) represents basis for all activities related to forest establishment (afforestation of available forest areas and abandoned agricultural land, burned areas, landfills, queries, protective belts) and also as a mean of help to natural regeneration (planting in cases of unsuccessful natural regeneration). There are estimates that available areas which could be brought to forest production amounts to more than 0,5 million ha in Croatia (Tijardović and Perić 2013). Even though natural regeneration of forest stands in Croatia is in principal good, there is evidence of growing problems (Oršanić et al 1996). The role of FRM is growing more important since natural regeneration of forest stands is prevented in many cases. It is also used in substitution/conversion activities which could be applied on areas under some kind of degradation stage (degradation stages marquise and garrigue 21%, coppices 22% – Croatian Forests L. t. d. 2006). Horticultural production also presents significance in the scope of urban forestry.

In addition, quality of FRM has direct influence on survival of planted seedlings and their growth success with the respect of its genetic traits (Perić et al 2009a). Increase of negative influences on natural ecosystems together with increase of public needs for all functions which forests provide open the space for more intensive nursery production in the future. These are the reasons why importance of biotechnological process of FRM production is consequently more pronounced. In addition, afforestation in the future should have more pronounced significance in the Croatian economy regarding the fact that besides utilization of forest products, ecological and other services forests also provide economical benefits in the frame of signed EU agreements.

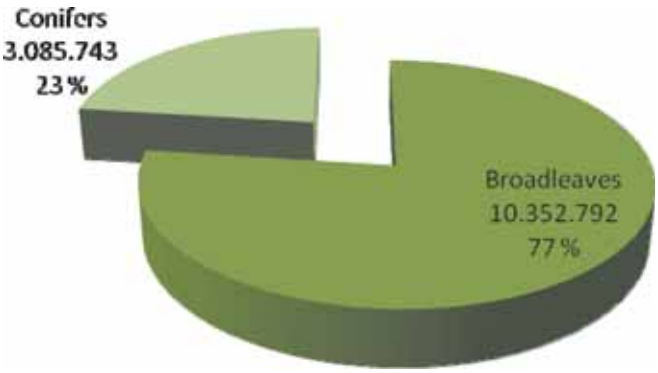
Materials and methods

Croatian Forest Research Institute (CFRI) was established during 1960-s with the aim of forest culture establishment with different purposes and was the leading institution in production of FRM in the beginning. The expert supervision is conducted in all registered nurseries for FRM production in Croatia and is appointed by the Ministry of agriculture. From the 1992 CFRI continually conducts expert supervision of nursery production which points to real needs not only in artificially established forest cultures but in natural stands as well. The data collected during expert supervision of FRM production which is conducted by CFRI was used for analysis of forest tree seedlings production.

Based on the reported production by individual nurseries monitoring of produced tree species by its origin, silvicultural methods, health condition and amount of produced plants was conducted annualy. These data are the basis for issuing related documents (Minute of conducted expert supervision and FRM List). According to already mentioned data on seedling production descriptive statistics was conducted for the past five year period (2008-2012). Five year monitoring data according to nurseries, tree species and cultivation methods fulfilled with overview on production trends from the beginning of expert supervision are significant for analysis of past production. In general, insight into production of FRM highlights the real needs not only connected with artificially establishment of forest cultures but also with related problems in natural regeneration. Estimations of future nursery production and all available areas suitable for afforestation will be harmonized with Strategy of rural development 2014-2020. Special overview was given to the total seedling production of all nurseries and cultivation methods in the year 2012 in addition with analyses of pioneer species which are suitable for establishment of forest cultures.

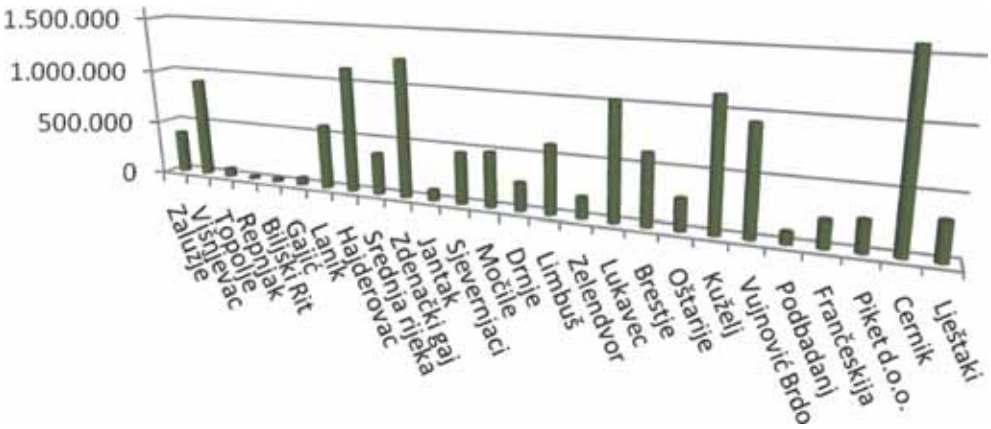
Research results

During 2012 in all observed nurseries in Croatia the total of 13.438.535 seedlings of both conifers and broadleaves, including different tree species, ages and cultivation method was produced. Data were acquired on the base of inventory list created on 30th of September of the current year. Out of total number of produced FRM the share of 77% refers to broadleaves while 23% refers to conifers. This is slightly better share for broadleaves if compared to 2011 (71,5%). Share of broadleaves and conifers RM is presented on Graph 1.



Graph 1 – Share of conifers and broadleaves in total production during 2012.

„Croatian forests" L. t. d. in 2012 participated with 97,6% in total production of FRM regarding the fact that CFRI stopped its production in the year 2010. Private nurseries have rather insignificant share in total production. The bigest share in production has Cernik nursery with 1.582.677 seedlings (Graph 2) on the area of Forest Administra-



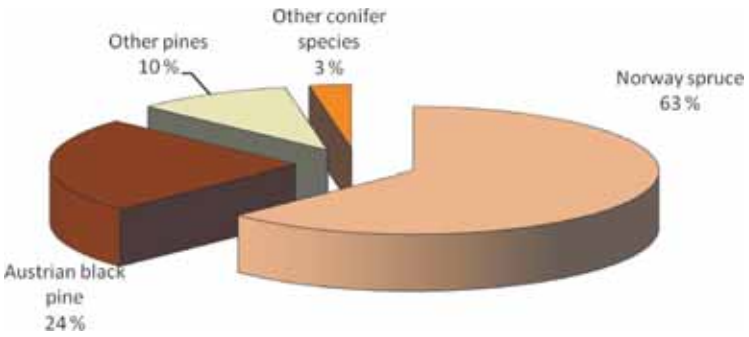
Graph 2 – Total production of broadleaves and conifer seedlings in the year 2012.

tion Office (AO) Nova Gradiška even though its production has decreased by one third in relation to the year 2010 (713.543 seedlings or 31% in relation to 2.296.220 seedlings). The largest production is noted in nurseries Hajderovac (1.132.948, area of Požega AO), Kuželj (1.132.625, area of Delnice AO), Zdenački Gaj (1.248.000, area of Bjelovar AO) and Lukavec (1.030.455, area of Zagreb AO). The lowest production is noted in the nurseries Repnjak (19.474, area of Osijek AO) and Biljski Rit (30.820, area od Osijek AO) while Travnik nursery, which in the latest years reported the lowest production in Croatia during 2012 stoped with its production.

According to nursery area the smallest nurseries from the beginning of expert supervision (preceding twenty years) are: Šubićevac, Voštarnica, III. Kono under the management of "Croatian Forests" L. t. d. with private nursery Lještaki L. t. d.. They all spread on the area covering less than 3 ha. The largest nurseries in Croatia in the last twenty years were Gaj (34,47 ha), Oštarije (32,56 ha), Lisičine (28,12 ha), Višnjevac (27,8 ha), CFRI (22,26 ha), Zalužje (22,21 ha) and Hajderovac (21,59 ha).

FRM production during 2012 in respect to 2011 was lower for 884.505 seedlings or 3,4% which presents the continuation of the negative trend of seedling production in Croatia (Orlić et al 2003, Orlić et al 2004, Perić et al 2005, Perić et al 2006, Perić et al 2007, Perić et al 2008, Perić et al 2009 b, Perić et al 2010, Perić et al 2011, Perić et al 2012). Decrease of total FRM production refers to conifer seedlings (5,8%) while the production of broadleaves seedlings has slightly increased (2,42%).

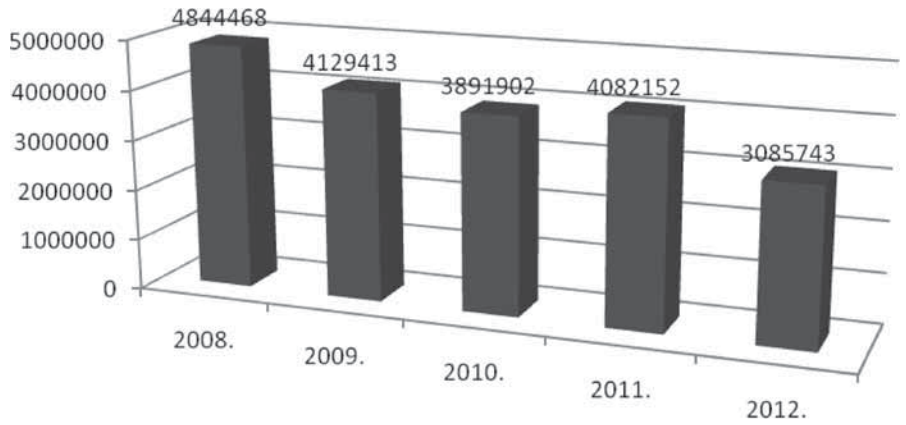
During 2012 3.085.743 of conifer seedlings were produced in total. Species with the biggest share in production of conifers remains to be Norway spruce (1.932.515 or 62,6%) with the similar share in total production of conifers as in the year before (2011). On the second place, according to production of seedlings is Austrian black pine (750.863). Even with smallest production this species has continued the increase of the share in total production (3%) and in conifer production (24,3%). These two species contribute with 87% in the total conifer production, while all other conifer species contribute with only 13% (Graph 3). Other conifer species



Graph 3 – Share of forest tree species in broadleaves production

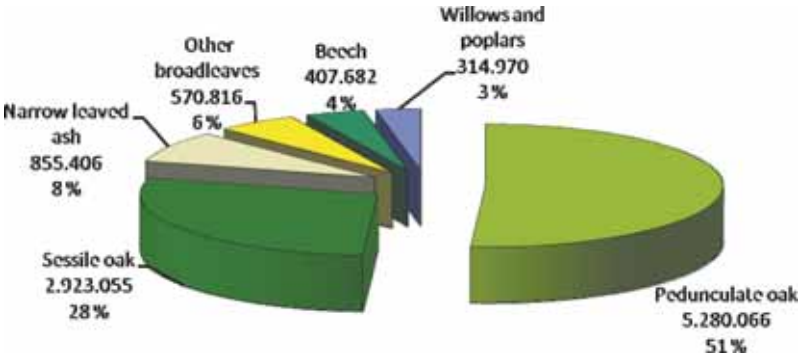
observed in the nursery production during 2012 are Scots pine (*Pinus sylvestris* L.), European larch (*Larix decidua* Mill.), Silver fir (*Abies alba* Mill.), Maritime pine (*Pinus pinaster* Ait.), Aleppo pine (*Pinus halepensis Miller.*), Cypress species (*Cupressus* sp.), Italian stone pine (*Pinus pinea* Engelm.) and Blue spruce (*Picea pungens* Engelm.).

The amount of produced conifer seedlings in the last five year period (2008-2012) spanned from 3.085.743 in 2012 up to 4.844.468 seedlings in 2008. 20.033.678 seedlings were produced in total in the examined five year period with 4.006.736 seedlings in average. Production of conifer seedlings during 2012 was below the average amount (lower for 920.993 seedlings). The conifer production trend was in the continuous decline from 2006. Negative trend has even pronouncedly continued in the 2012. Conifer production trend in the researched period is presented on Graph 4.



Graph 4 – Production of conifer seedlings in Croatia in all registered nurseries in the period from 2008-2012

During 2012 the total of 10.352.792 broadleaves seedlings was produced. The share of broadleaves seedlings in the total production amounts to 77% (Graph 1). Broadleaves species with the biggest share can be observed on Graph 5. The biggest share in production has the Pedunculate oak (*Quercus robur* L., 5.280.066 seedlings or 51%). Then follow, similar to preceding years, Sessile oak (*Quercus petraea* Matt., 2.923.055 or 28,2%), Narrow-leaved ash (*Fraxinus angustifolia* Vahl., 855.406 or 8%) and Common beech (*Fagus sylvatica* L., 407.682 or 4%). The share of individual broadleaves species can be observed on Graph 5. All other broadleaves contribute with only 6%, while willows and poplars contribute to broadleaves production with 3% (314.970). Production of Pubescent oak (*Quercus pubescent* Willd.), Turkey oak (*Quercus cerris* L.), Holm oak (*Quercus ilex*), Black locust (*Robinia pseudoacacia* L.), Hornbeam (*Carpinus betulus* L.); Black alder

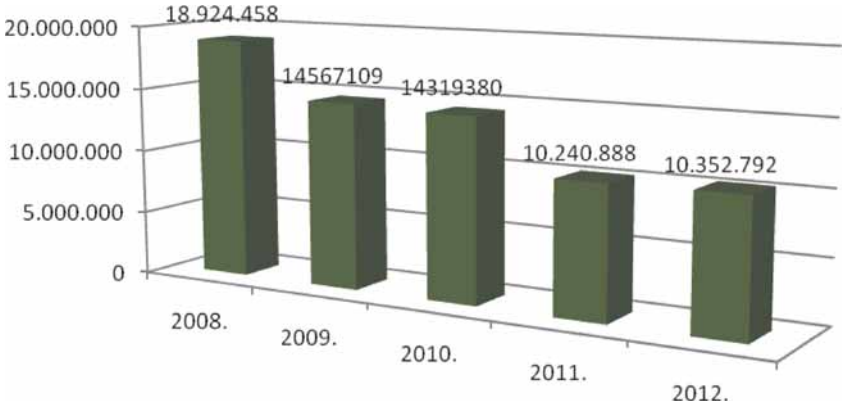


Graph 5 – Share of production of individual tree species in production of broadleaves forest reproductive material in Croatia

(*Alnus glutinosa* (L.). Gaertn.), Wild cherry (*Prunus avium* L.) and Black walnut (*Juglans nigra* L.) was also marked.

Nurseries with the biggest production of broadleaves in 2012 were Cernik, Zdenački Gaj and Hajderovac which are accompanied with Lukavec. Nurseries with the biggest production of conifer seedlings are Kuželj (area of Delnice AO) and Vujnović Brdo (Gospić AO).

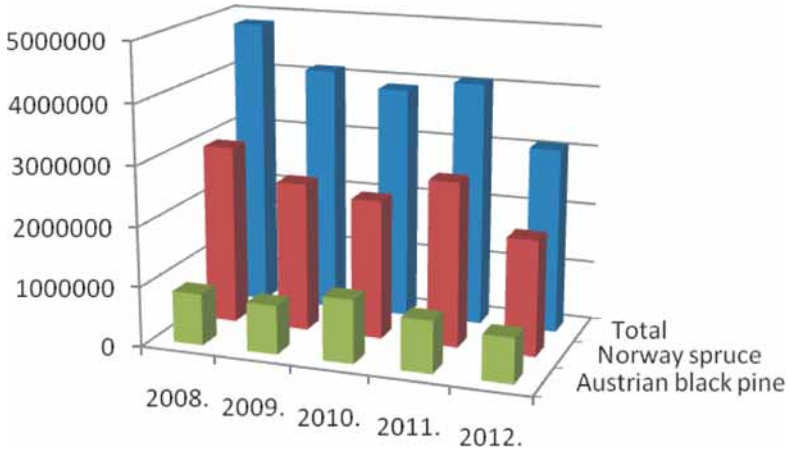
The amount of produced broadleaves in the last five year period (2008-2012) spanned from 10.240.888 in 2011 up to 18.917.606 seedlings in 2008. The average production amounts to 13.680.925 seedlings. Seedling production of broadleaved species during 2012 marks slight increase in the respect to the year 2011 (111.904), but it is lower for 3.328.133 seedlings than the average (lower for 417.352 seedlings). Broadleaves production trend is in continuous decline in the last five year period what can be observed on Graph 6 and follow the long-term trend



Graph 6 – Production of broadleaves seedling in Croatia in all registered nurseries in the period from 2008-2012

(Perić et al 2008, Perić et al 2009 b, Perić et al 2010, Perić et al 2011, Perić et al 2012).

Production of most relevant conifer species during 2011 and 2012 is presented on Graph 7. It is evident that the production varies. In 2012 conifer species with the biggest share in total conifer production were again Norway spruce (*Picea abies* Karst.) and Austrian black pine (*Pinus nigra* L). Total of 1.932.515 seedlings of Norway spruce and 750.863 seedlings of Austrian black pine was produced. In the last five year period in Croatia was produced 12.473.891 seedlings of N. spruce and 4.376.183 seedlings of A. black pine in total.



Graph 7 – Production of Norway spruce and Austrian black pine seedlings in Croatia in all registered nurseries in the period from 2008-2012

The amount of produced Norway spruce seedlings continually decreases in the last five years with slight increase in 2011. The span of seedling production in preceding five years was between 2.991.401 seedlings in 2008 to the lowest amount of 1.932.515 seedlings in 2012. Production of Austrian black pine varies and spannes from 1.065.204 seedlings in 2010 to only 750.863 in 2012.

Average production in the researched period for Norway spruce is 2.494.778 seedlings and for Austrian black pine 875.237. Production of N. spruce during 2012 is lower then the average amount (for 22,5%) while in the case of A. black pine is higher for 10,2%.

The share of Pedunculate oak seedlings in total production varies from year to year and it amounts around half of broadleaves production. It spanned from 51% (2012) to 57% (2008). Slight decrease is marked for the production in the last three years.

During 2012 314.970 seedlings of willows and poplars were produced, out of which 286.070 poplar seedlings and 28.900 willow seedlings. In the last five year period total of 1.275.816 seedlings of both species were produced (1.217.600 of poplar seedlings or 95,4% and 58.216 of willow seedlings or 4,6%). The production of poplars increases annually up to 2009, while in 2010 and 2011 it slightly drops. The largest production is marked in 2012 with the amount of 286.070 seedlings, while the lowest was in the year 2011 (220.653). Production trend of willows shows decrease up to 2012 while significant increase was marked in relation to 2011 (26.600 seedlings or around 12 times). The lowest amount of produced willow seedlings was observed in the years 2010 and 2011 with the amount of 2.300. There is in total 1.275.816 of willow and poplar seedlings produced in the last five year period (1.217.600 of poplar seedlings or 95,4%, and 58.216 of willow seedlings or 4,6%).

Discussion and conclusions

Decrease in the number of forest nurseries was observed for the last twenty year period (from the beginning of expert supervision) for the whole country. According to Strategy of nursery production of "Croatian Forests" L. t. d. which is a public cooperation that manages almost all nurseries in Croatia there will be a further decrease of the number of nurseries. The reorganisation of nursery management will be carried out from 2014 and will incorporate, besides reduction in the number of nurseries specialisation of remaining nurseries by tree species and their bioclimatic orientation.

In addition, analyses of FRM production from the beginning of expert supervision (Perić et al 2009 a) showed a decrease with some fluctuations (Orlić and Perić 2002, Orlić et al 2003, Orlić et al 2004, Perić et al 2005, Perić et al 2006, Perić et al 2007, Perić et al 2008, Perić et al 2009 b, Perić et al 2010, Perić et al 2011, Perić et al 2012). Broadleaves production in the last five year period also continues with its decline. In the last twenty years their production fluctuates, following negative production trend especially in the earlier years. Production of conifer seedlings, which are mainly used for forest culture establishment, also fluctuates and show decrease.

If production of individual tree species is taken into account there is different situation. Tested poplar and willow clones in 2012 show increase together with Norway spruce (62,2 %) which are species suitable for afforestation activities. Nevertheless, in comparison with the beginnings of early days of expert supervision the total amount of 3.085.743 seedlings of Norway spruce presents great decline. The data of pioneer species production is in accordance with the amount of area afforested annually which also decreases (Oršanić 2003). Significance of pioneer spe-

cies and other species with pioneer features is great for afforestation of uncovered forest and suitable agricultural areas where those species have ameliorative function of preparing site for climax species. In addition, FRM of pioneer species is used for conversion of degraded forest sites (Dubravac et al 2006, Španjol et al 2009) where high forests of climax species present not only ecological and productive maximum of the specific area but also a mean of fire management (Raftoyannis et al 2013). Fire events are estimated to become more frequent in the scope of changing climate especially in Mediterranean areas (Williamson et al 2005). Its significance is even more highlighted in the scope of fulfilment of Kyoto protocol obligations which Republic of Croatia has signed and ratified (obtaining the carbon credits) or as a fulfilment of national or EU strategies for fighting climate change and fulfilment of renewable energy strategies (e. g. fossil fuel substitution effects, etc.) (Tijardović et al 2013).

Some alochtonous tree species possess great wood production potential (e. g. Douglas fir, European larch, Weymouth pine) and could be used for establishment of new forest cultures and plantations of various purposes. Nevertheless, their production has not even in 2012 found its place in seedlings production in Croatia. In addition, rather insufficient share of nursery production refers to planting material of rare and endangered species which also possess important place in forestry.

The share of Pedunculate oak seedlings in the total production varies from year to year but it refers to half of the broadleaves production. If we take into consideration that this is a climax tree species which should be regenerated naturally these values are high especially in relation to production of pioneer species suitable for afforestation activities (establishment of new forests). Significant share of climax species in total production of FRM in 2012, in general, highlights serious problems during natural regeneration of forest stands. In changed site conditions it is mandatory to give as better starting conditions to the seedlings after out planting as possible, so it is highly recommended to produce quality planting material of appropriate age. It is also recommended that with adequate seedling selection its morphological features should be balanced with specific site conditions for which seedlings have been acquired. In relation to that investment in modernisation of nursery production presents permanent and priority task because whole production process have direct influence on seedling quality which are delivered to the field.

Twenty year expert supervision together with analysis of obtained data on FRM production in nurseries across Croatia point to existence of bias between production and real needs in praxis. Lack of appropriate planning results with bias of production goals and out planting needs especially related to individual species and seedling type. In addition, better harmonization of seedlings production and needs in other economy sectors (e. g. for recultivation of queries, landfills, recultivation of areas along highways, ...) is also needed. In general, planning efforts are needed

not only to avoid bias between production and needs on the field but also that planting material should not be stored in nursery longer then is biologically possible. In this way the cost of nursery production could be lowered and planting material of optimal age could be delivered. The growing production of FRM could present high positive impact on economic growth as well, especially in rural areas. Possibilities of FRM production in the future are connected with export because currently there is a high capacity for production in forest nurseries in Croatia. However, lack of appropriate export strategy also presents problems if planned nursery production and production efficiency are to be taken into account.

There are current activities on FRM production enhancement in Croatia. For example, harmonisation of legislative on FRM with the aims of EU (new Low on FRM), preparation of new Strategy of nursery production, establishment of new Seed stand and clonal seed orchards registry, opened Seed and gene bank (CRFI), new Croatian brands which creates recognition of Croatian planting material in whole Europe (e.g. Slavonian oak), etc. Even though there are current efforts on FRM production enhancement due to ever growing change of natural conditions we recommend more attention to be paid to follow significant changes in the technologies of seedlings production in the world and changes which are based on the principles of sustainability and climatic and ecological issues. Also, implementation of new scientific knowledge and innovations in whole production process together with new biotechnological applications such as tree improvement, inoculation of seedlings with micorisa fungi, hydro gels, new and improved fertilizers, improved containers and root trainers, etc. could serve as a means of adaptation and mitigation measures for fighting climate changes and changes in other site conditions.

Therefore, possibilities for nursery production in the future are greater than they are in presence and could secure increase of production in general and increase of production of individual tree species in particular (e. g. pioneer species and rare and endangered species which are used for natural stands improvement). Unfortunately, growing threats and negative forces upon forest ecosystems are predicted to increase. Problems with regeneration of natural stands could also be expected in the future (Oršanić et al 1996) while there are predictions on further increase of climate changes with more frequent natural disturbances and fire events which also present threat to natural ecosystems. For example Gardiner et al 2011 predict increase in the number of storms for Europe but they highlight even stronger impact on forest ecosystems through increase in storms intensity.

FRM possesses great importance for sustainable forest management (e. g. Colombo 2001) regardless the natural or artificially established forests are taken into the account. Therefore, due to all services and benefits which forests provide to people and to economy sectors in Croatia its importance together with importance of related FRM should be taken into the account more seriously in the future.

References

1. COLOMBO S. J., 2001: How to improve the quality of broadleaved seedlings produced in tree nurseries. In: Proceedings of the conference „Nursery production and stand establishment of broad-leaves to promote sustainable forest management“, Rome, Italy, May 7-10, 2001.
2. CROATIAN FORESTS LTD. 2006 Forest management plan 2006 – 2015.
3. DUBRAVAC, T., S. DEKANIĆ, 2009: Struktura i dinamika sječe suhih i odumirućih stabala hrasta lužnjaka u Spačvanskom bazenu od 1996. do 2006. godine. Šumarski list CXXXIII(7-8), p 391-405.
4. DUBRAVAC, T., D. BARČIĆ, Ž. ŠPANJOL, B. VRBEK, V. ROTH, S. DEKANIĆ, 2006: Natural reforestation of aleppo pine (pinus halepensis mill.) stands after forest fire. Forest Ecology and Management 234S (2006) S180-S207.
5. OECD official webpage: www.oecd.org (accessed on 12th November 2013)
6. EFI ATLANTIC, 2013: Planted forests are a vital resource for future green economies. Summary report of the 3rd International Congress on Planted Forests. Available at: www.efiatlantic.efi.int/portal/events/past_events/2013/icpf/ (Accessed on 12th November 2013).
7. GARDINER B., K. BLENNOW, J.-M. CARNUS. Š- FLEISCHER, F. INGEMARSON, G. LANDMANN, M LINDNER, M. MARZANO, B. NICOLL, C. ORAZIO, J.-L. PEYRON, M.-P. RAVIRON, M.-J. SCHELHAAS, A. SCHUCK, M. SPIELMANN, T. USBECK, 2011: Destructive Storms in European Forests: Past and Forthcoming impacts. European Forest Institute, Final Report to European Commission – DG Environment, 2011.
8. ORLIĆ S., S. PERIĆ, 2002: Proizvodnja šumskih sadnica u Hrvatskoj 1997-2001. godine. Radovi 37 (2), p 211-223.
9. ORLIĆ S., S. PERIĆ, T. DUBRAVAC, V. ROTH, Ž. OREŠKOVIĆ, 2003: Izvješće o obvezatnom stručnom nadzoru nad proizvodnjom šumskih sadnica u rasadnicima «Hrvatskih šuma» d.o.o. Zagreb i rasadniku Hrvatskog šumarskog instituta za 2003. godinu.
10. ORLIĆ S., S. PERIĆ, T. DUBRAVAC, V. ROTH, Ž. OREŠKOVIĆ, 2004: Izvješće o obvezatnom stručnom nadzoru nad proizvodnjom šumskih sadnica u rasadnicima «Hrvatskih šuma» d.o.o. Zagreb i rasadniku Hrvatskog šumarskog instituta za 2004. godinu.
11. ORŠANIĆ M., S. MATIĆ, I. ANIĆ, 1996: O izboru vrsta drveća za obnovu sastojina hrasta lužnjaka zahvaćenih sušenjem. Hrvatsko šumarsko društvo, Skrb za Hrvatske šume od 1846.-1996, p 127-132.
12. ORŠANIĆ M 2003 Pošumljavanja, sadašnje stanje i perspektive u globalnom razvoju. Šum. list CXXVII (2003) , pos. izd.; 35-40
13. PERIĆ S., 2001: Prvi rezultati o produkciji drvne mase u pokusu provenijencija hrasta lužnjaka (*Quercus robur* L.) u Hrvatskoj. Znanost u potrajnom gospodarenju hrvatskim šumama. Scientific book, p 223-232.
14. PERIĆ S, A. JAZBEC, J. GRAČAN, 2003: Grouping of Pedunculate oak provenances on the basis of biomass by applying cluster analysis. 25th International Conference Information Technology Interfaces ITI 2003, June 16-19 2003, Cavtat, Croatia, p 217-221.
15. PERIĆ S., S. ORLIĆ, M. IVANKOVIĆ, 2004: Growth of six coniferous species in different bioclimates in Croatia. Ekologia-Bratislava, 23 (1), p 86-98.
16. PERIĆ S., M. TIJARDOVIĆ, M. ORŠANIĆ, J. MARGALETIĆ, 2009a: Rasadnička proizvodnja i važnost šumskog reprodukcijskom materijala u RH. Rad. Hrvat. Šumar. inst. 44 (1), p 17–27.
17. PERIĆ S., T. DUBRAVAC, V. ROTH, Ž. OREŠKOVIĆ, 2005: Izvješće o obvezatnom stručnom nadzoru nad proizvodnjom šumskih sadnica u rasadnicima «Hrvatskih šuma» d.o.o. Zagreb i rasadniku Hrvatskog šumarskog instituta za 2005. godinu.

18. PERIĆ, S., T. DUBRAVAC, V. ROTH, Ž. OREŠKOVIĆ 2006: Izvješće o obvezatnom stručnom nadzoru nad proizvodnjom šumskih sadnica u rasadnicima «Hrvatskih šuma» d.o.o. Zagreb i rasadniku Hrvatskog šumarskog instituta za 2006. godinu.

19. PERIĆ, S., T. DUBRAVAC, V. ROTH, Ž. OREŠKOVIĆ 2007: Izvješće o obvezatnom stručnom nadzoru nad proizvodnjom šumskih sadnica u rasadnicima «Hrvatskih šuma» d.o.o. Zagreb i rasadniku Hrvatskog šumarskog instituta za 2007. godinu.

20. PERIĆ, S., M. TIJARDOVIĆ, T. DUBRAVAC, V. ROTH 2008: Izvješće o obvezatnom stručnom nadzoru nad proizvodnjom šumskih sadnica u rasadnicima «Hrvatskih šuma» d.o.o. Zagreb i rasadniku Hrvatskog šumarskog instituta za 2008. godinu.

21. PERIĆ S., M. TIJARDOVIĆ, T. DUBRAVAC, V. ROTH, 2009b: Izvješće o obvezatnom stručnom nadzoru nad proizvodnjom šumskih sadnica u rasadnicima «Hrvatskih šuma» d.o.o. Zagreb i rasadniku Hrvatskog šumarskog instituta za 2009. godinu.

22. PERIĆ S., M. TIJARDOVIĆ, T. DUBRAVAC, V. ROTH, 2010: Izvješće o obvezatnom stručnom nadzoru nad proizvodnjom šumskih sadnica u rasadnicima «Hrvatskih šuma» d.o.o. Zagreb i rasadniku Hrvatskog šumarskog instituta za 2010. godinu.

23. PERIĆ S., M. TIJARDOVIĆ, T. DUBRAVAC, V. ROTH, 2011: Izvješće o obvezatnom stručnom nadzoru nad proizvodnjom šumskih sadnica u rasadnicima «Hrvatskih šuma» d.o.o. Zagreb i rasadniku Hrvatskog šumarskog instituta za 2011. godinu.

24. PERIĆ S., M. TIJARDOVIĆ, T. DUBRAVAC, V. ROTH, 2012: Izvješće o obvezatnom stručnom nadzoru nad proizvodnjom šumskih sadnica u rasadnicima «Hrvatskih šuma» d.o.o. Zagreb i rasadniku Hrvatskog šumarskog instituta za 2012. godinu.

25. PERRY DAVID A., 1998: The scientific basis of forestry. Annual Review of Ecology and Systematics, Vol 29, 435-466.

26. RAFTOYANNIA Y., S. NOCENTINI, E. MARCHI, S. R. CALAMA, G. C. GARCIA, I. PILAS, S. PERIC, P. J. AMARAL, S. C. MOREIRA-MARCELINO, M. COSTA-FERREIRA, E. KAKOURIS, M. LINDNER, 2013: Perceptions of forest experts on climate change and fire management in European Mediterranean forests. Journal of Biogeosciences and Forestry

27. ŠPANJOL, Ž., HRŠAK, V., BARČIĆ, D., ANČIĆ, M., DUBRAVAC, T., ROSAVEC, R., ORŠANIĆ, M., 2009: Pine reforestation of degraded sites on the island of Rab, Croatia. Plant Biosystems

28. TIJARDOVIĆ M, PERIĆ S, 2013: Croatian Norway spruce cultures in European concept of culture management. *Periodicum biologorum, Vol. 115, No. 3,* p 355-361.

29. TIJARDOVIĆ M., J. PARVIAINEN, S. PERIĆ, 2013: Carbon Storage Potential of Forest Land: A Comparative Study of Cases in Finland and Croatia. SEEFOR, Vol 4, No 1, p 23-34.

30. WILLIAMSON T. B., J. R. PARKINS, B. L. MCFARLANE, 2005: Perceptions of climate change risk of forest ecosystems and forest-based communities. Forestry Chronicle 81, p 710-716.

Higher Education for the Requirements of Industrial Production: European Experience and Croatian Challenges

Vladimir Mrša*

Minireview

University of Zagreb, Faculty of Food Technology and Biotechnology,
Pierottijeva 6, 10000 Zagreb, Croatia

The times of rapid development of technology require adequate rethinking of principles on which modern education, and in particular higher education has to be based to prepare young population for ever more challenging requests of their future jobs. The reforms of high education take place world-wide with different success. How is Croatian high education system coping with the present requirements and what are the chances of our high education institutions in a competitive European high education area? This article tries to sum up briefly some of the particular questions raised, as well as to propose at least some of the answers to pave a path to what one might see as a more efficient and more successful high education area.

Introduction

European higher education (HE) undergoes a process of convergence towards a rather homogenous education area in which national systems are easily comparable and, although still maintaining some individual specificity, all share the same basic principles. This trend is mainly a result of increasing communications among European higher education institutions (HEI) leading to adoption of common standards and a sort of „optimization" of HE systems in different countries. Besides, it is also a result of a necessity for comparability of different systems as a prerequisite for increasing mobility of students and teachers. Cooperation between HEI significantly exceeds simple exchange of teaching stuff, or collaboration on joint research and projects towards joint study programmes leading to either joint, or double diplomas. With this respect it is easy to envision a fully homogeneous study/research area in which students would enrol different courses at different universities moving from one town or country to another each year, or even semester, shaping up their education according to their own interests and affinities. Obviously, such homogenous high education system would require not only comparable administrative procedures and learning outcomes, but it would also imply comparable quality of HEI. This, in turn, would require decreasing differences in invest-

*Corresponding author: vmrsa@pbf.hr

ments in high education in different European countries. In today's European Union with marked differences in BDP among member countries, current trends in the development of high education seem like a logical up-grade of institutions and education processes in western and northern countries with higher BDP. In the same time, it presents a great challenge for eastern and south-eastern countries, including Croatia. Expensiveness of modern high education originating in the requirement for more practical training involving expensive equipment and facilities on one hand, and in the ever increasing fraction of students in the young people population on the other, threats to create a gap between countries with higher and those with lower investments in high education, jeopardising the overall homogenisation process and restricting it to only the EU countries with high BDP. Clearly, such a threat is more than evident in the Croatian HE and is particularly emphasized in the times of recent economic crisis when cutting research and education funds has a much more dramatic effect in countries with lower HE budgets. Can a country with a rather low BDP and a clearly underdeveloped HE system still keep pace with much richer European countries and, if so, what should stakeholders in the education process do to achieve this? In particular, how is this going to reflect on the studies of engineering disciplines which per definition require more resources and are therefore significantly more expensive than studies of humanistic or social sciences? Certainly, it would require a substantial reform of HEI and the system itself, starting with the objective identification of present weaknesses, involving a sincere wish to achieve changes, as well as efficient mechanisms for their implementation. HE system of every country acts in a given organisational framework in which study curricula are implemented using education resources, and conforming to quality standards assured by a quality assurance component of the system. For a true fundamental reform Croatian HE has to achieve improvements in all these constituent factors. Therefore, in the next paragraphs a short description of the state-of-the-art will be given with possible directions Croatian HE area could assume to meet the challenges of the reforms required to keep the pace with HE in Europe and provide the fundament for a better society of Croatia's tomorrow.

Organisational framework

Croatian HE system belongs to so called binary education systems. This means that the Croatian HEI comprise universities, as well as vocational universities and vocational schools. Besides, two types of study programmes are thought at our HEI, university programmes and the vocational programmes. University programmes are implemented exclusively at universities, while vocational programmes can be found mainly at vocational universities and vocational schools, but some are performed at universities, as well. What is the European experience with binary sys-

tems? Are they accepted widely or not and, consequently, should Croatia keep it or not? The analysis of European HE systems shows that the answer to the question cannot be found in European trends or experiences. Namely, 14 European countries, among which some known of undisputed quality in high education, such as Germany, Denmark, Belgium or the Netherlands, also have binary systems. In contrast, 14 European countries, among which UK, Norway, France, Ireland or Spain with equally respected HE, have only universities. The data clearly indicate that there is no correlation between the type of the HE system and its quality. As far as the number of HEI is concerned, there are 10 universities, 15 vocational universities, and 28 vocational schools in Croatia. The number is higher than in most European countries of comparable size and it is worth noting that it doubled in the period between 2003 and 2010. With respect to the owner, 7 universities, 12 vocational universities and 3 vocational schools are public, while 3 universities, 3 vocational universities and 25 vocational schools are private. Thus, most universities and vocational universities belong to the public sector, while the majority of vocational schools are private. Out of about 153.000 Croatian students three quarters study at universities while one quarter study at vocational schools and vocational universities. As far as the study programmes are concerned, altogether 1351 program is offered by Croatian HEI which is significantly higher than in European HE systems of comparable sizes. Somewhat less than 50% of programmes are in the fields of social sciences and humanistic, about one quarter belong to technical and biotechnical sciences, while the rest comprise programmes in natural sciences, biomedicine and arts. It is worth noting that vocational schools, particularly private ones, mainly offer programmes in the field of economics while at universities and vocational universities the programme offer is distributed more evenly. This short analysis shows that the system maintains a large number of study programmes with small number of students per programme (113 students in average) which makes HE costly and extremely resource demanding. Thus a process of rationalization of the number of study programmes seems reasonable. On the other hand, both the number of programmes and the number of students in the field of social and humanistic sciences seems too high, while the number of students studying programmes in the STEM area seems too small for the society requiring significant increase in industrial production and, consequently, more highly educated experts in these fields. This points out that a culture of promotion of STEM disciplines already at the primary and secondary school levels is necessary to create enough students interested in the corresponding studies. Rather low number of students interested in STEM area is actually a Europe-wide problem, but countries like Germany, Switzerland or the Scandinavian countries already started various programmes with the aim of raising interest for mathematics and engineering at early education levels.

Entrance quota at Croatian HEI is very high and in fact exceeds the number of students leaving the four year secondary schools by as much as 23%. Such high

quota is to a great deal a result of the lack of any systematic estimate of real social demand for high educated population. Thus the quota is suggested by HEI themselves and it can easily be observed that in some study programmes the quota exceeds the realistic capacities of these institutions. As expected, this occurs mainly with programmes which do not require practical forms of training and certainly decreases the quality of courses. Therefore, reliable mechanisms of estimation of real social demands for different expert profiles are required, and the entrance quota has to be consolidated with the demands and the capacities of HEI. Certainly, decrease of entrance quota has to be accompanied with better employability of students finishing secondary schools, particularly of the vocational type.

Particular emphasis should be pointed towards the part time students. In the existing HE system the study of part time students is burdened with a number of obstacles. Part time students do not have student benefits other students enjoy like food or accommodation subventions, they have to pay for the scholarship participation, and in some cases are treated in the same way as the regular students. In this way part time studying is not enhanced but sometimes used simply to increase the quota and the HEI's income. Part time studying is particularly important in the education of engineers where new skills have often to be developed in parallel with fulfilling the commitments of the existing employment. Therefore, including part time studies in the process of life-long learning with students whose status, including the scholarship coverage, is not different from that of the regular students seems like a logical solution. Part time studies should not differ in learning outcomes, or content of study programmes, but rather in the pace of studying which should be individually adjusted to student's out-of-study commitments. HEI should put additional effort in creating study groups of part time students, particularly in the STEM area.

Study curricula

Croatia has started the process of transformation of our study programmes according to the Bologna principles in the year 2004 and the first students enrolled newly created studies in the academic year 2005/06. Main goals of the reform were making studies less expensive by shortening their average duration, making students better prepared for the labour market demands by dividing the high education cycle into undergraduate (bachelor), and graduate (master) studies with more practical skills involved in curricula, and to decrease drop-out by promoting teaching in smaller groups with a more individual approach to students. Now, eight years after the implementation of the Bologna reforms, these goals have been achieved in different European countries with very different success. In some countries, like Germany or Italy, employability of bachelors is very low, while in some other, like the

Netherlands it amounts to over 50%. Similarly, some countries, particularly in the Eastern Europe still have problems with high drop-out rates, some do not have sufficient practical training as a part of the regular curricula and many countries have achieved only a modest decrease in the average study duration. Obviously, Bologna reform has presented a huge twist in the European HE and different countries have had different capacities and different financial resources to cope with a radical change. In Croatia, we cannot be satisfied with the achievements of the Bologna goals either and one can assume two major reasons for this. First, the reform has been started without the required preparations. The new curricula have been created without defining connections with the learning outcomes, neither of individual courses, nor of whole study programmes. The preparations of the Croatian Qualification Framework (CQF) have started much later, thus generations of students have already completed their high education according to the Bologna studies without clear definitions of their professions and their position at the labour market. Finally, no additional resources have been foreseen for the new studies although it has been very reasonable to predict that teaching in smaller groups with individual approach requires more teachers, facilities and equipment. This particularly stands for the studies in the STEM area, although in these studies and individual teacher-student relation has always been assumed. The second problem in implementation of the Bologna reform was the fact that it was carried in the bottom-up way with no strict organizational framework defined by the state, in spite of the fact that it has clearly been a national reform. As a result, each HEI approached the transformation process individually, with different specific goals, different comprehension of the necessity of a change, and consequently, with different enthusiasm. Some HEI devoted much effort to improve their studies, while some other remained at the level of strictly superficial changes without tackling the essence of the reform. The differential approach to modernization of studies created differences in students' satisfaction, as well, being very low at HEI which have not taken the Bologna process seriously enough. Besides, the Bologna reform has been implemented at most HEI as a one-time intervention and not as a process. Thus, no further evaluations and adjustments have been undertaken in the meantime, although it is very obvious that some need to be done.

In this year Croatia has brought the Law on Croatian Qualification Framework and the work on the creation of the catalogue of professions and required competences proceeds but it can be foreseen that it will take at least two years to be completed. In the meantime HEI should clearly define the learning outcomes of their studies. This process can be expected, among other benefits, to point out which study programmes create same or similar profiles of experts which in turn could serve as the basis for the already mentioned rationalization and for better interconnections among HEI. Besides, measures have to be taken to assure that the learning outcomes defined by the study programme indeed lead to competences guaranteed by the diploma issued by the HEI leading to a proper positioning not only within the

CQF but, through its complementarity with the European Qualification Framework (EQF), at the European labour market, as well.

Several issues have to be addressed in the process of rethinking Bologna reform. In redesigning study programmes according to the Bologna principles many HEI, particularly faculties, adopted the strategy of creating strong bachelor studies. This was usually done by condensing contents of courses of previous undergraduate studies which lasted four to five years to three to four years of BSc Bologna studies. This kind of strategy brought to several negative consequences. First, at many BSc courses number of ECTS points awarded to the student is lower than his actual workload. Actually, student's total workload per semester sometimes seems to be much higher than 30 ECTS. Besides, the relations among different courses in terms of awarded ECTS are often incorrect. All this speaks in favour of a necessity for redistribution of ECTS, not only among BSc courses but also between BSc and MSc studies since at MSc studies the situation seems to be opposite. Second problem appearing with the implementation of Bologna studies is that most faculties introduced formal obstacles in vertical mobility of students from BSc to MSc studies. This kind of measures is implemented mainly to ensure that students studying vocational studies in the first cycle (BSc) would not be able to proceed to university MSc studies in the second cycle. Since, as already mentioned, university bachelor studies are often much more difficult than the corresponding master studies, only the administrative measures can prevent the students' opportunistic strategy of by-passing the university BSc studies and replacing them with vocational studies. Besides, practically all students achieving the BSc level enrol and subsequently complete further MSc studies leading to complete lack of bachelors at the labour market. It has to be noted, however, that the latter situation results also from the general lack of jobs caused by the economic crisis, but sometimes also from the fact that bachelor studies often do not end with concrete competences required by the employers but are rather meant to be the preparative step for further study.

High education resources

In terms of resources required for efficient and high quality HE we can distinguish among human resources, equipment, facilities, and financial means.

In Croatian HE area there has been an on-going discussion on whether we have enough, too many, or too few teachers. The analysis of the teacher/student ratio shows that this parameter is in average acceptable at universities (21), but not at vocational universities and vocational schools (41). Since the share of students studying at the latter institutions is about 25%, the overall number of teachers falls

below the recommended limit ratio of 1:30. However, it should be mentioned that the 1:30 ratio may be applicable for studies in social and humanistic sciences, but not for other studies like those in the STEM area. Taking into account that the number of students will decrease in the coming years due to Croatian demographic trends which are significantly negative, and due to expected lowering of entrance quotas which are generally too high and exceed the public needs, particularly in some fields, the number of HE teachers seems satisfactory. Changes are, however, needed in the structure, and partly also in the quality of human resources. Current practice is that a university teacher gets promoted into a higher position after he has spent a certain period of time at the lower position providing he has fulfilled certain formal criteria like the number and quality of published papers etc. The promotion is not linked with the actual requirement for the position the teacher is elected to. Since the formal conditions for promotions are generally quite low, teachers advance fast and reach the level of full professor at an early age resulting in a "reversed pyramid" of positions in many organizational units. Although a transition to a new system with defined hierarchy of positions in HEI is the only possible outcome, and is the way HEI in practically all other European countries are organized, it will clearly take time and should only be set as a long-term goal. Besides the election criteria seem quite formal and some of them do not include the quality parameters. Therefore, they also require adequate up-grade. In the evaluation of teachers' performance in STEM area it seems very important to put adequate significance on patented and not only paper-published results to encourage applied aspects of research.

Adequate equipment is a prerequisite of efficient HE, particularly in the STEM area. The on-going paradox is that HEI, not only in Croatia but world-wide, cannot afford the same level of modern instruments as the employers. This results with the situation in which they are in a position to educate experts to use sophisticated equipment at their future working positions but in the same time possess, and therefore organize courses using much less sophisticated apparatus. Keeping in mind expenses generated by purchasing, maintaining and using up-to-date equipment there seem to be two possible ways by which HEI could at least partially solve this problem. One is to establish interconnections between HEI and employers in which students would be allowed to get acquainted with the instruments used by employers' R&D, or analytical departments and do a part of their training, like practical placement or diploma work, there. The other involves collaboration among HEI which could create joint platforms for particular research operations which require expensive equipment. Such approach would bring significant rationalization in expenditures but would also enable better training of stuff operating the equipment and more efficient usage of instruments.

It is hardly necessary to mention that a modern HE system requires an efficient ICT network. IC technologies provide means for improvement of education process it-

self, like teaching tools and communication platforms for teachers and students, but they are also of extreme importance for management of HEI and the system in total. Efficient management relies on data which have to be correct, up-dated, and handy and easy to reach and use. In the Croatian HE there are two main communication systems, one maintained by the Croatian Agency for Science and Education ("MOZVAG"), and the other by Croatian universities ("ISVU"). Although both systems have been up-graded and improved in the last years, they still do not contain all the required data and cannot perform all operations required by HEI. Besides, they are only partially interconnected and the exchange of data between the systems is difficult if possible at all. It seems reasonable to assume that their synthesis in one system which would communicate with other data resources, like those of the Ministry of Science, Education and Sport, the Croatian Bureau of Statistics and other relevant institutions would provide a better tool for management processes in the Croatian HE area.

A number of Croatian HEI work in old and in many ways inadequate buildings which sometimes makes competitiveness in the European HE area impossible. Although building new university campuses requires significant investments, European funds (particularly ESI) could provide for the required finances. It has to be mentioned, however, that new investments in buildings have to be carefully planned and that the new facilities have to be rationally constructed to avoid high maintenance costs. Besides, organization of university campuses only has sense if it is accompanied by functional cross-linking among faculties occupying the campus space.

Adequate financing is a prerequisite of the HE system development. In times of economic crisis when in most European countries budgets for research and education are restricted, spending money rationally is more important than ever. Croatian HE area has already started a process of restructuring of the model of financing public HEI. The model should be transformed into a program agreement based system in which basically the state contracts HEI to perform certain tasks and achieve certain goals in high education. Transformation of the existing financing system is a complex process which has to proceed slowly and cautiously avoiding risks of jeopardising the vital functions of HEI. Program agreements should comprise all issues connected with the education process including research as the integral component of high education, internationalization process, quality assurance, student standard etc. They should comprise financing all types of HEI, all types of study programmes (university, vocational) and all kinds of students (full time, part time). Financing should be sufficient to enable positioning of Croatian HEI in the competitive European HE area, and efficient to ensure maximal result of invested means. It has to be mentioned that research and high education in Croatia are financed mainly by public funds while the private investment is almost negligible. It is not realistic to expect a significant increase in budgeting of this area by the state,

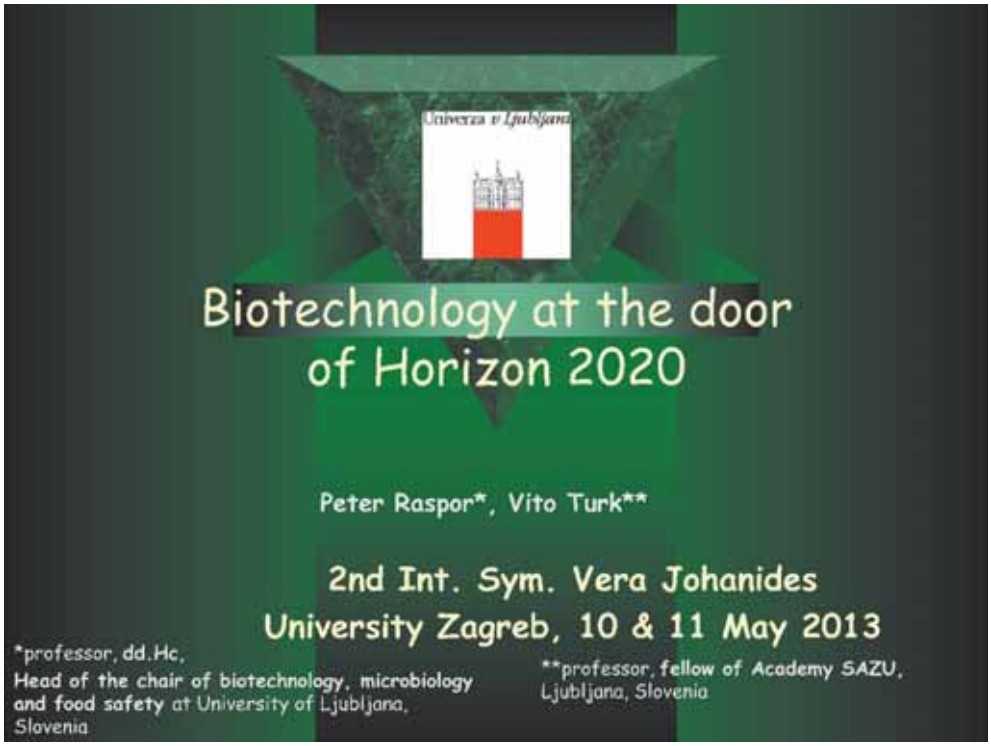
thus better connections between HEI and industry or other employers is of utmost importance. Only clear comprehension of necessity of implementation of knowledge in creating new value-added products and achieving global competitiveness of our companies can lead to joint projects between production companies and academia. In other words, the only way HEI can significantly increase their incomes is by an influence on the increase of income of their partners in the production sector by putting more emphasis on knowledge based production of goods and services. To facilitate these processes interest networks should be created comprising HEI, research institutes, production companies, state bodies and other stakeholders in a particular research area.

Quality assurance in high education

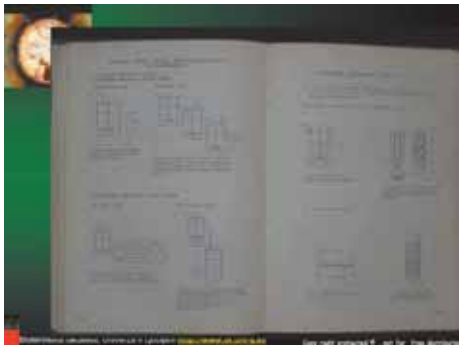
Quality assurance mechanisms are probably the most important component of a HE system. Most surveys of European HEI show that there is no apparent correlation between their quality and their type, size, or geographical position, but that the quality relies on efficient quality assurance. In Croatia, the mechanisms of HE quality assurance are organized by the Croatian Agency for Science and High Education and they are regulated by the corresponding legislative framework which defines the criteria a HEI should meet to acquire the accreditation of their study programs. The system is rather new and was implemented in the present form in 2010. Although there is a general opinion among the HE stakeholders that the system is operational, certain improvements should be made, particularly in the process of initial accreditation which is rather lose and is followed by a long period of 5 years before the first reaccreditation occurs. An efficient ICT system which would allow continuous following of the quality parameters would therefore be more adequate for efficient quality management. Besides, putting the ultimate importance on quality of HE implicates that HEI should only differ in their type and study programs but not in their quality and that they should therefore all fulfil the same quality criteria. Finally, one should conclude that the final goal of the quality system improvement should be development of the quality culture in which striving for excellence would come inherently from the HE stakeholders and would not have to be imposed by external institutions. Although reaching this level today seems unrealistic to many, it provides the only possible way in positioning of Croatian HEI in the European HE surrounding.

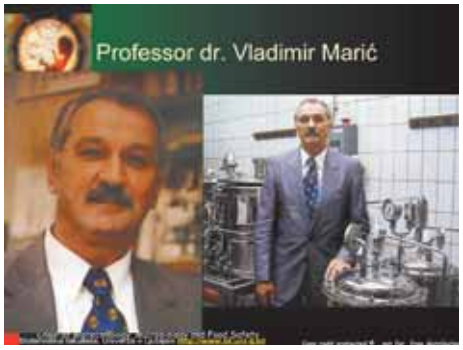
Part II

Presentations

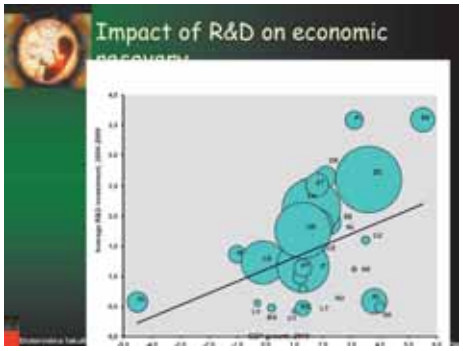

Univerza v Ljubljani
Biotechnology at the door of Horizon 2020
Peter Raspor*, Vito Turk**
2nd Int. Sym. Vera Johanides
University Zagreb, 10 & 11 May 2013
*professor, dd.Hc,
Head of the chair of biotechnology, microbiology and food safety at University of Ljubljana, Slovenia
**professor, fellow of Academy SAZU, Ljubljana, Slovenia

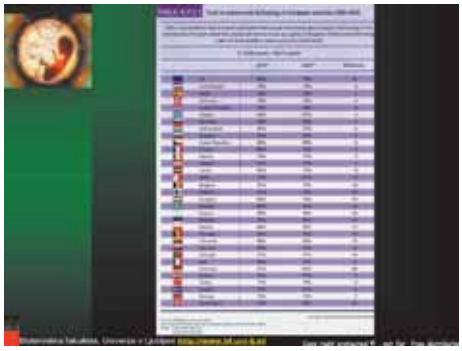

Professor dr. Vera Johanides

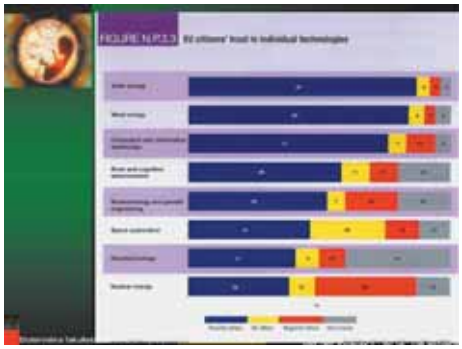



Professor dr. Vladimir Marić


The Multiannual Financial Framework 2014-2020
1. Smart & inclusive growth (€491bn)
Horizon 2020
2. Sustainable growth, natural resources (€383bn)
3. Security and citizenship (€18.5bn)
4. Global Europe (€70bn)
5. Administration (€62.6bn)
Total: € 1,025bn


Impact of R&D on economic





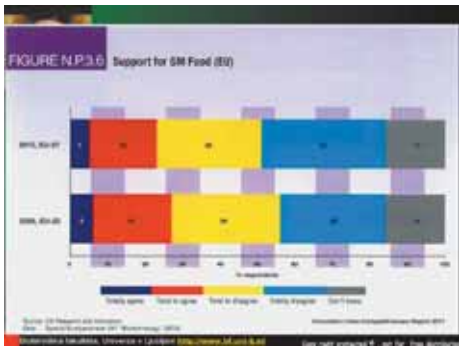
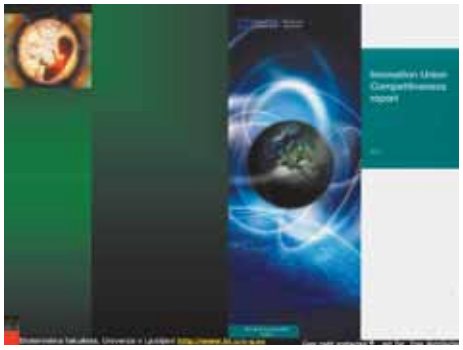
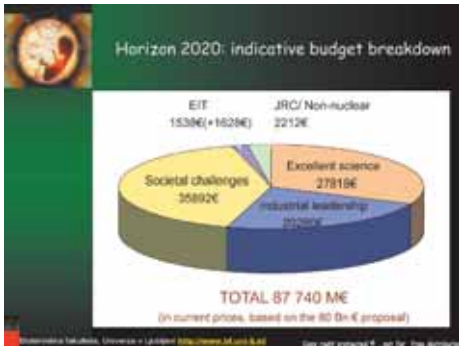


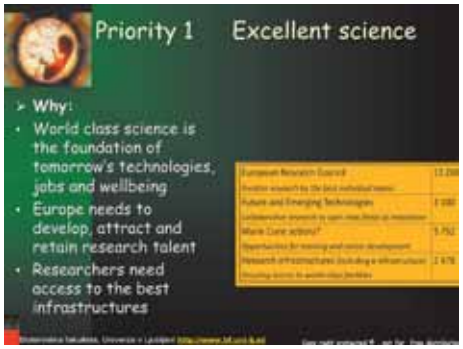
FIGURE N.P.3.6 Support for GM Food (EU)




What is Horizon 2020
What's new


Three priorities of Horizon 2020:
1 Excellent science
2 Industrial leadership
3 Societal challenges


Horizon 2020: indicative budget breakdown
EIT
1538€(+1626€)
JRC/ Non-nuclear
2212€
Societal challenges
35892€
Excellent science
27818€
TOTAL 87 740 M€


Priority 1 Excellent science
Why:
World class science is the foundation of tomorrow's technologies, jobs and wellbeing
Europe needs to develop, attract and retain research talent
Researchers need access to the best infrastructures


Priority 2 Industrial leadership
Why:
Europe needs more innovative SMEs to create growth and jobs
Strategic investments in key technologies (e.g. advanced manufacturing, micro-electronics) underpin innovation across existing and emerging sectors
Europe needs to attract more private investment in research and innovation


Priority 3 Societal challenges
Why:
EU policy objectives (climate, environment, energy, transport etc) cannot be achieved without innovation
Breakthrough solutions come from multi-disciplinary collaborations, including social sciences & humanities
Promising solutions need to be tested, demonstrated and scaled up


Horizon 2020 and partnering
Public-Private Partnerships (PPPs):
Through Joint Technology Initiatives or other formal structures (§ 187)
Via contractual arrangements, which provide inputs for workprogrammes
Only when criteria met, e.g. clear commitments from private partners
Public-Public Partnerships (P2Ps):
Through "ERA-Nets" for topping up individual calls/ actions (replacing current ERA-Net, ERA-Net Plus, Inco-Net, Inno-net)
Through participation in joint programs between Member States (§ 185.)
Supporting agendas of Joint Programming Initiatives when in line with Horizon 2020
Only when criteria met, e.g. financial commitments of participating countries
European Innovation Partnerships
Not funding instruments, but for coordination with broader policies and programmes


Commission's proposal
SINGLE SET OF RULES
for Horizon 2020
SIMPLIFICATION


Rules for Participation: what's new? (1)
1. A SINGLE SET OF RULES
Adapted for the whole research and innovation cycle
Covering all research programmes and funding bodies
Aligned to the Financial Regulation, coherent with other new EU Programmes
2. ONE PROJECT - ONE FUNDING RATE
Maximum of 100% of direct costs (except for actions close to market, where a 70% maximum will apply)
Indirect eligible costs: a flat rate of 20% of direct eligible costs
3. SIMPLE EVALUATION CRITERIA
-Excellence - Impact - Implementation (Excellence only, for the ERC)
4. NEW FORMS OF FUNDING aimed at innovation: pre-commercial procurement, inducement prizes, dedicated loan and equity instruments
5. INTERNATIONAL PARTICIPATION: facilitated but better protecting EU interests


Rules for Participation: what's new? (2)
6. SIMPLER RULES FOR GRANTS: broader acceptance of participants accounting practices for direct costs, flat rate for indirect costs, no time-sheets for personnel working full time on a project, possibility of output-based grants.
7. FEWER, BETTER TARGETED CONTROLS AND AUDITS
Lowest possible level of requirements for submission of audit certificates without undermining sound financial management;
Audit strategy focused on risk and fraud prevention.
8. IMPROVED RULES ON INTELLECTUAL PROPERTY
Balance between legal security and flexibility;
Tailor-made IPR provisions for new forms of funding;
A new emphasis on open access to research publications.
Beyond the Rules: further simplified provisions in the Grant Agreement and implementing procedures to facilitate access to Horizon 2020 (eg. common IT platform).


Widening participation
by unlocking R&I potential of cohesion countries
Principle of excellence: continue to allocate funding on the basis of competitive calls, selecting only the best projects.
Clear division of labour between cohesion policy and Horizon 2020.
Cohesion policy: support for regions in building up their research and innovation capacity.
Horizon 2020: widen participation, better coordination between the two Union funding programmes, support policy learning reforms.
Accompanying measures in Horizon 2020 to ensure that excellence prevails wherever it exists, including: twinning, ERA chairs, support for access to international networks, development of smart specialisation strategies.


Specific proposals on broadening access to research and innovation excellence in Horizon 2020


Societal Challenge on Inclusive, innovative and secure societies / Part 6.1.4. Closing the research and innovation divide in Europe (I)
Significant regional disparities across Europe in research and innovation performance need to be addressed. Measures will aim at unlocking excellence and innovation and will be distinct, complementary and synergistic with policies and actions of the Cohesion policy Funds. They include:
Linking emerging institutions, centres of excellence and innovative regions in less developed Member States to international leading counterparts elsewhere in Europe:
measures include teaming of excellent research institutions and less developed regions, staff exchanges, expert advice and assistance and the development of joint strategies for the establishment of centres of excellence that may be supported by the Cohesion policy funds in less developed regions.
Building links with innovative clusters and recognising excellence in less developed regions, including through peer reviews and awarding labels of excellence to those institutions that meet international standards, will be considered.


Societal Challenge on Inclusive, innovative and secure societies / Part 6.1.4. Closing the research and innovation divide in Europe (II)
Establishing 'ERA Chairs' to attract outstanding academics to institutions with a clear potential for research excellence, in order to help these institutions fully unlock this potential and hereby create a level playing field for research and innovation in the European Research Area.
This will include institutional support for creating a competitive research environment and the framework conditions necessary for attracting, retaining and developing top research talent within these institutions.

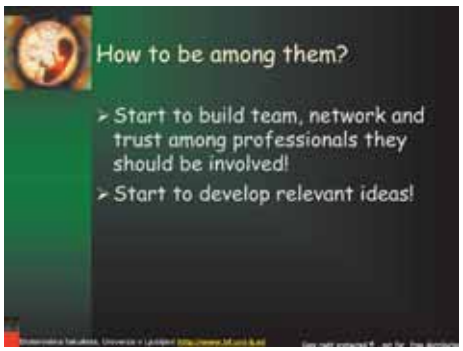

Societal Challenge on Inclusive, innovative and secure societies / Part 6.1.4. Closing the research and innovation divide in Europe (III)
Supporting access to international networks for excellent researchers and innovators who lack sufficient involvement in European and international networks. This will include support provided through COST and National Contact Points.
Supporting the development and monitoring of smart specialisation strategies. A policy support facility will be developed and policy learning at regional level will be facilitated through international evaluation by peers and best practice sharing.


What next?


The first work programmes will also support the groundwork for future Strategic Programmes.
Personalising health and care
Sustainable food security
Blue growth: unlocking the potential of the oceans
Smart cities and communities
Competitive low-carbon energy
Energy Efficiency
Mobility for growth


The first work programmes will also support the groundwork for future Strategic Programmes.
Waste: a resource to recycle, reuse and recover raw materials
Water innovation: boosting its value for Europe
Overcoming the crisis: new ideas, strategies and governance structures for Europe
Disaster-resilience: safeguarding and securing society, including adapting to climate change
Digital security


How to be among them?
"European bioeconomy challenges: Food security, sustainable agriculture and forestry, marine and maritime and inland water research" are among major concerns shared by European citizens and therefore this area is one among the six priority challenges selected for funding.


How to be among them?
Start to build team, network and trust among professionals they should be involved!
Start to develop relevant ideas!


To work hard and to believe in your ideas and trust to your team


Katedra za
Biotehnologijo, Mikrobiologijo in varnost živil,
Biotehniška Fakulteta,
Univerza v Ljubljani,
Slovenija
2012


Hvala lepa!
Univeristy of Ljubljana, Biotehnical faculty, Chair of biotechnology, microbiology and food safety
Jamnikarjeva 101
1001 LJUBLJANA
SLOVENIJA
Tel.: +386 (0)1 423 11 61
Fax: +386 (0)1 256 57 82

Green biotechnology in agriculture – food, biofuel and recombinant DNA technologies.

Domagoj Šimić[1], Krešimir Dvojković[2], Aleksandra Sudarić[3].

[1] Agricultural institute Osijek, Department for Maize Breeding and Genetics
[2] Agricultural institute Osijek, Department for Breeding and Genetics of Small Cereal Crops
[3] Agricultural institute Osijek, Department for Breeding and Genetics of Industrial Plants

Green biotechnology

- Various definitions
- Wikipedia; Pejić, Kereša, Šimić, Buhiniček: Biotechnology applied to agricultural processes expanding to

1. gen**omics** ← PRECURSOR: Selection and domestication of plants (breeding)
2. recombinant gene technologies - applied particularly in plant production → designing of transgenic (GM) plants

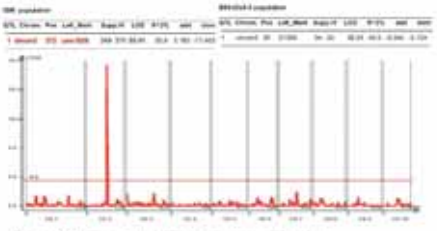
Agricultural Institute Osijek

- Genomics - routinely adopted for developing new varieties of maize, wheat and soybean
- **Ionomics** - detection of chromosome segments in maize associated with biofortification traits in grain to increase the concentrations of bioavailable minerals Fe and Zn → food biotechnology

Agricultural Institute Osijek

- **Ionomics** → high-throughput genetic analysis of ion concentrations of 22 elements in maize (mostly beneficial, but also toxic)
- Concentrations of almost all elements controlled by multiple genes
- Cadmium (Cd) concentration controlled only by 1, 2 genes. Confirmation and validation in two maize populations (Sorić et al., 2009)

Quantitative trait loci analysis for **cadmium** accumulation in maize leaf



→ Candidate gene approach; no positional cloning

Cadmium in food

- Higher Cd concentrations in wheat and soybean grain (EFSA – European Food Safety Authority, 2012) ← fertilization in agriculture (Cd is by-product in mineral fertilizers)
- **Cd** can cause kidney failure and has been statistically associated with an increased risk of cancer
- decreasing of **Cd concentration** in crops by recombinant DNA technologies?

Genetically modified (GM) crops for food

- Green biotechnology = recombinant DNA technologies in crop plants = GM crops for food

30 years of GM crops
GM CROPS:
PROMISE & REALITY
A *Nature* special issue,
2 May 2013 nature.com/gmcrops

"the speed and precision that they offer over traditional breeding techniques made them indispensable 30 years ago. They still are today."

Food to fuel

Shifting the paradigm in agriculture to biofuel production seems to be promising for green biotechnology

- Crop plants - not domesticated for modern biofuel production → energy conversion efficiency?
- → quickest, most efficient way to convert to biofuel feedstocks is biotechnologically

GM crops for fuel

Recombinant DNA technologies for increasing **Cd concentration** in maize → phytoremediation of Cd contaminated soil because of intensive fertilization

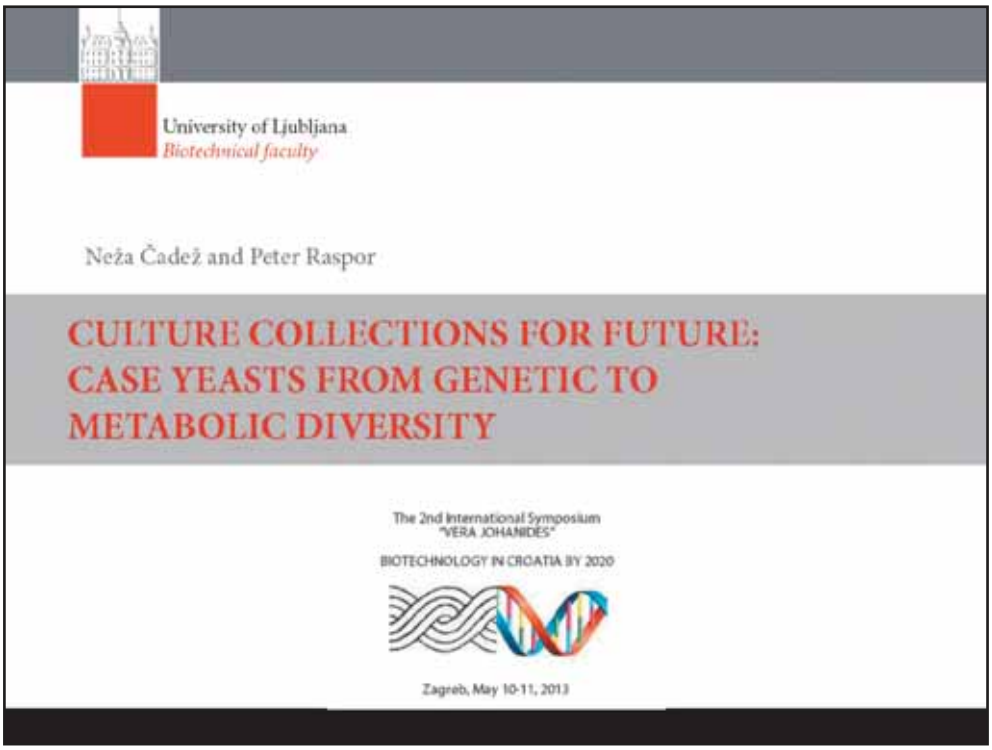
- more fertilization → more biomass and/or higher yields for biofuel production
- advocating safe and appropriate use of technology and environmental protection

New GM crops for food and fuel

- "editing a plant's own version, rather than bringing in an external gene", *Nature*, 2013
  = cisgenesis ⇔ transgenesis (EU)

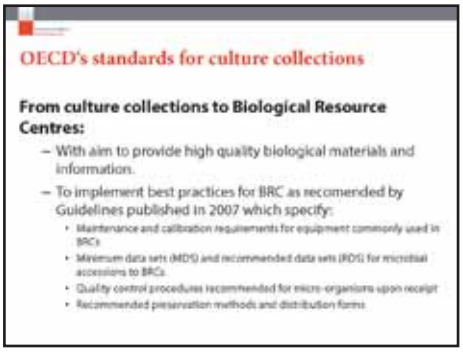
- Equivalent to traditional plant breeding but recombinant DNA technologies are applied
- Maize Cd genes for Cd accumulaton in maize

Mario Franić talk - tomorrow


University of Ljubljana
Biotechnical faculty
Neža Čadež and Peter Raspor
CULTURE COLLECTIONS FOR FUTURE: CASE YEASTS FROM GENETIC TO METABOLIC DIVERSITY
The 2nd International Symposium "VERA JOHANIDES"
BIOTECHNOLOGY IN CROATIA BY 2020
Zagreb, May 10-11, 2013


Culture collections – link to microbial diversity
1 % of all described species (Mršić, 1997)


The legal base for Culture collections
Convention on Biological Diversity
OECD
The objectives of the CBD are:
1. Conservation of biological diversity.
2. Sustainable use of its components.
3. Fair and equitable sharing of the benefits arising from commercial and other utilization of genetic resources.
„Biological resource centres are an essencial part of the infrastructure underpinning life sciences and biotechnology".


OECD's standards for culture collections
From culture collections to Biological Resource Centres:
– With aim to provide high quality biological materials and information.
– To implement best practices for BRC as recomended by Guidelines published in 2007 which specify:
• Maintenance and calibration requirements for equipment commonly used in BRCs
• Minimum data sets (MDS) and recommended data sets (RDS) for microbial accessions to BRCs.
• Quality control procedures recommended for micro-organisms upon receipt
• Recommended preservation methods and distribution forms

Estimated costs to implement OECD standards

Item	Costs (EURO €)
Initial costs	55 000
Advisor / Trainer	
Continuously surveillances costs	55 000
Audits	
Evaluation Customer Satisfaction	
3% additional workload for staff	
Consultation	
Total 1st year	110 000

Estimated costs to comply with OECD standards

Estimation of Costs

Item	Costs (EURO€)
Cultures duplicate storage	15 000
Personnel (3)	23 000
External audit	17 000
Other direct costs	5 000
Total	95 000

Current types of culture collections

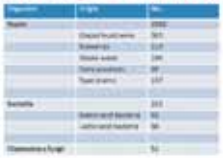
Public repositories

- Large, diverse collections
- Type strains of most (all) species
- Reference strains
- Patent repository

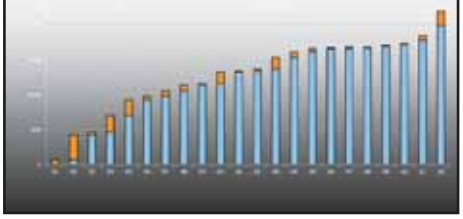
„Boutique collections"

- Narrower range of species
- Historic speciallity: wine, brewing, ecology
- Broadening services to capture additional funding.

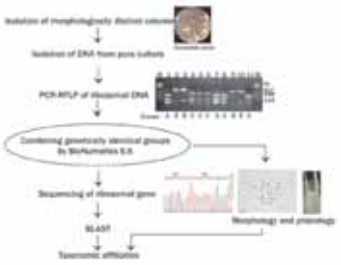
Slovenian Collection of Industrial Microorganisms (ZIM)



Acquiring new strains from establishment of ZIM in 1991



Current identification scheme



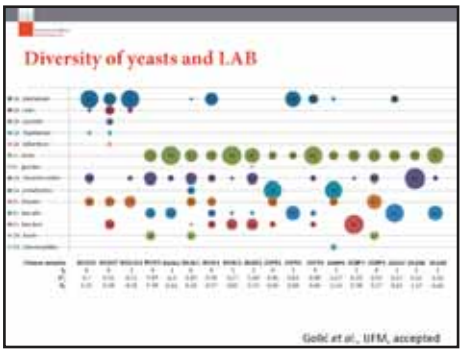
Research projects

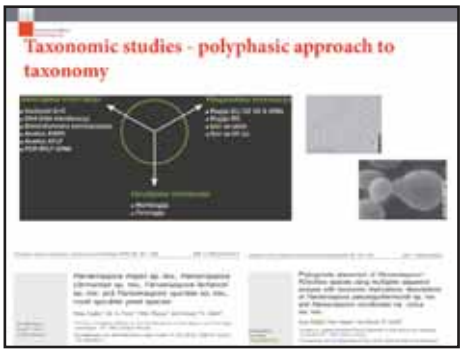
Population dynamics of fermented foods.
Ecological factors affecting yeast diversity.
Yeasts as biocontrol agents.
Diversity of yeasts and their impact on food quality.

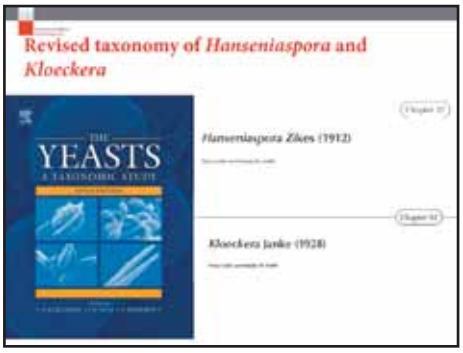
Evaluation of microbial diversity in traditional cheeses from Serbia and Croatia

PSALAB No. 195

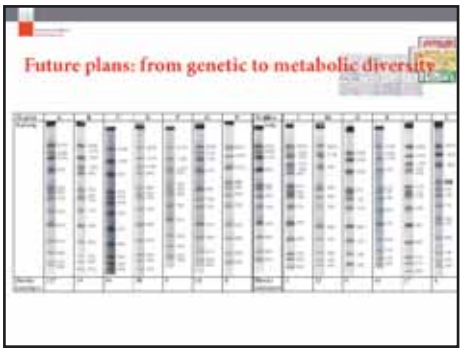
White-pickled cheese — Mountain Djelatica, Despotovac, Serbia


Diversity of yeasts and LAB
Golić et al., UFM, accepted


Taxonomic studies - polyphasic approach to taxonomy


Revised taxonomy of Hanseniaspora and Kloeckera
THE YEASTS
A TAXONOMIC STUDY
Hanseniaspora Zikes (1912)
Kloeckera Janke (1928)

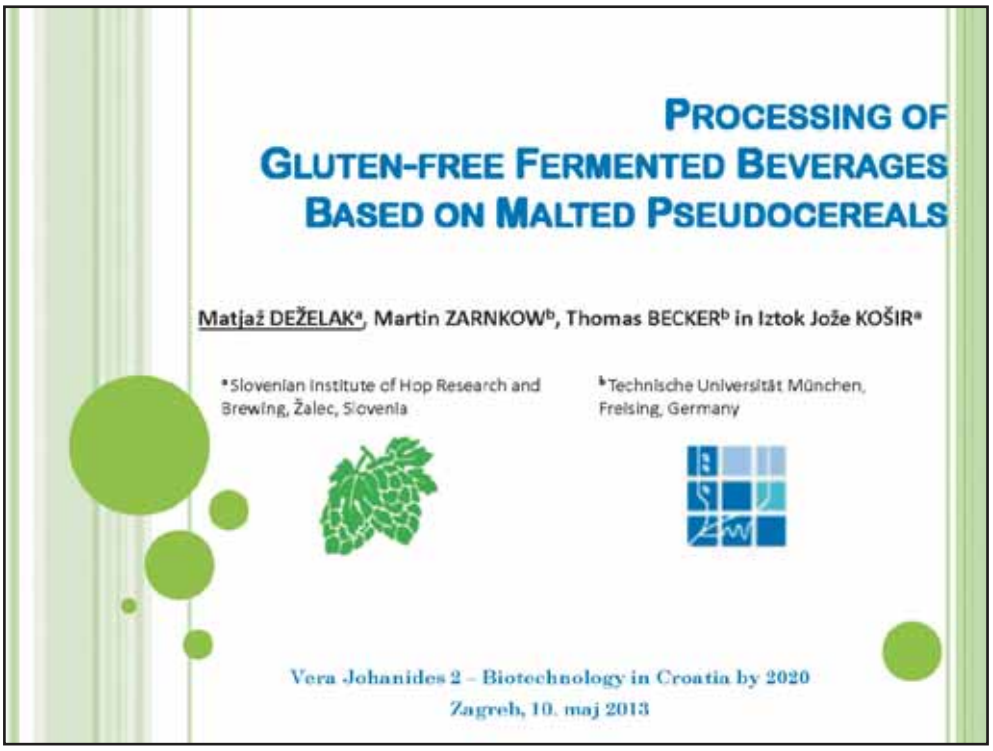

Hot-spot of unexplored yeast species diversity – Slovenian olive oil
Not to be described: Kurssiella sp. nov. and Saturnispora sp. nov. all from Slovenian olive oil


Future plans: from genetic to metabolic diversity


Acknowledgement


Acknowledgement

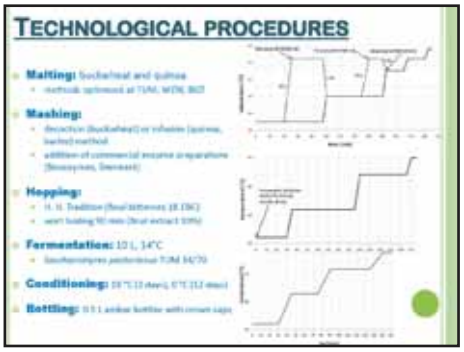

THANK YOU FOR YOUR ATTENTION!
HVALA LEPA ZA VAŠO POZORNOST!

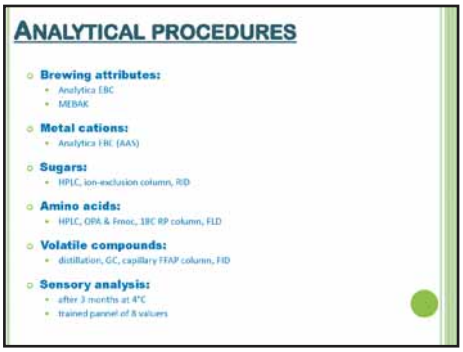

PROCESSING OF
GLUTEN-FREE FERMENTED BEVERAGES
BASED ON MALTED PSEUDOCEREALS
Matjaž DEŽELAK[a], Martin ZARNKOW[b], Thomas BECKER[b] in Iztok Jože KOŠIR[a]
[a]Slovenian Institute of Hop Research and Brewing, Žalec, Slovenia
[b]Technische Universität München, Freising, Germany
Vera Johanides 2 – Biotechnology in Croatia by 2020
Zagreb, 10. maj 2013

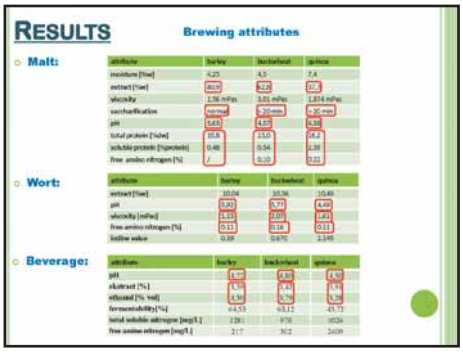

INTRODUCTION
Celiac disease

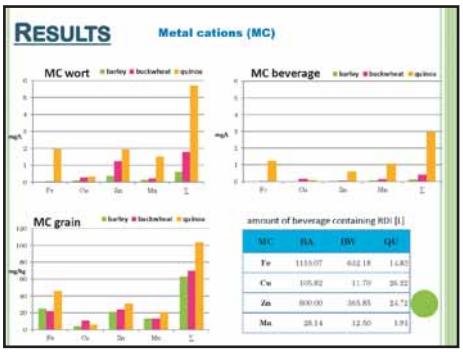

INTRODUCTION
Functional foods
Buckwheat
Quinoa


RESEARCH IDEA & HYPOTHESES
Beer:
Idea:
Hypotheses:

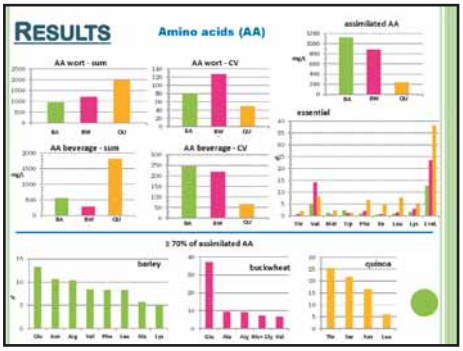

TECHNOLOGICAL PROCEDURES
Malting:
Mashing:
Hopping:
Fermentation:
Conditioning:
Bottling:

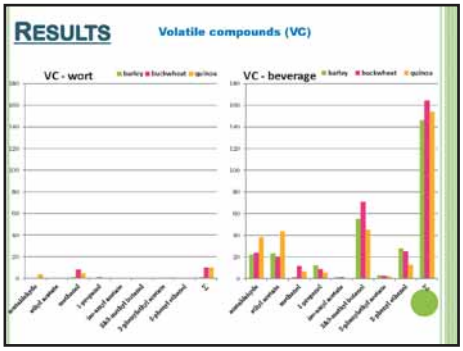

ANALYTICAL PROCEDURES
Brewing attributes:
Analytica EBC
MEBAK
Metal cations:
Analytica EBC (AAS)
Sugars:
HPLC, ion-exclusion column, RID
Amino acids:
HPLC, OPA & Fmoc, 18C RP column, FLD
Volatile compounds:
distillation, GC, capillary FFAP column, FID
Sensory analysis:
after 3 months at 4°C
trained pannel of 8 valuers

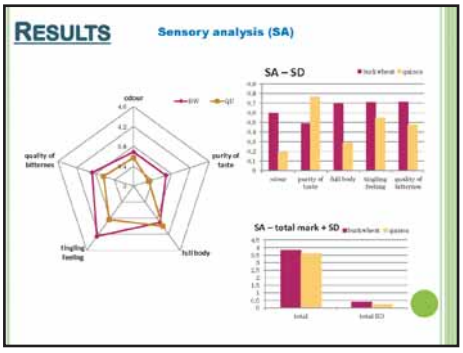

RESULTS
Brewing attributes
Malt:
Wort:
Beverage:

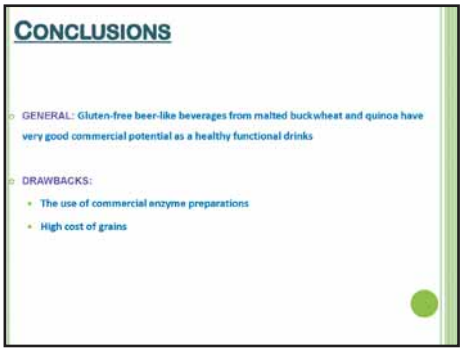

RESULTS
Metal cations (MC)
MC wort
MC beverage
MC grain
amount of beverage containing RDI [l]

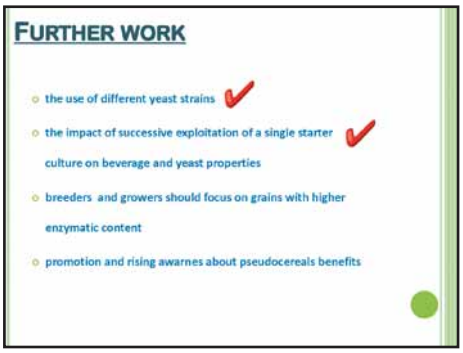

RESULTS
Sugars (OH)
OH wort
OH beverage


RESULTS
Amino acids (AA)
AA wort - sum
AA wort - CV
assimilated AA
AA beverage - sum
AA beverage - CV
essential
≥ 70% of assimilated AA
barley
buckwheat
quinoa


RESULTS
Volatile compounds (VC)
VC - wort
VC - beverage

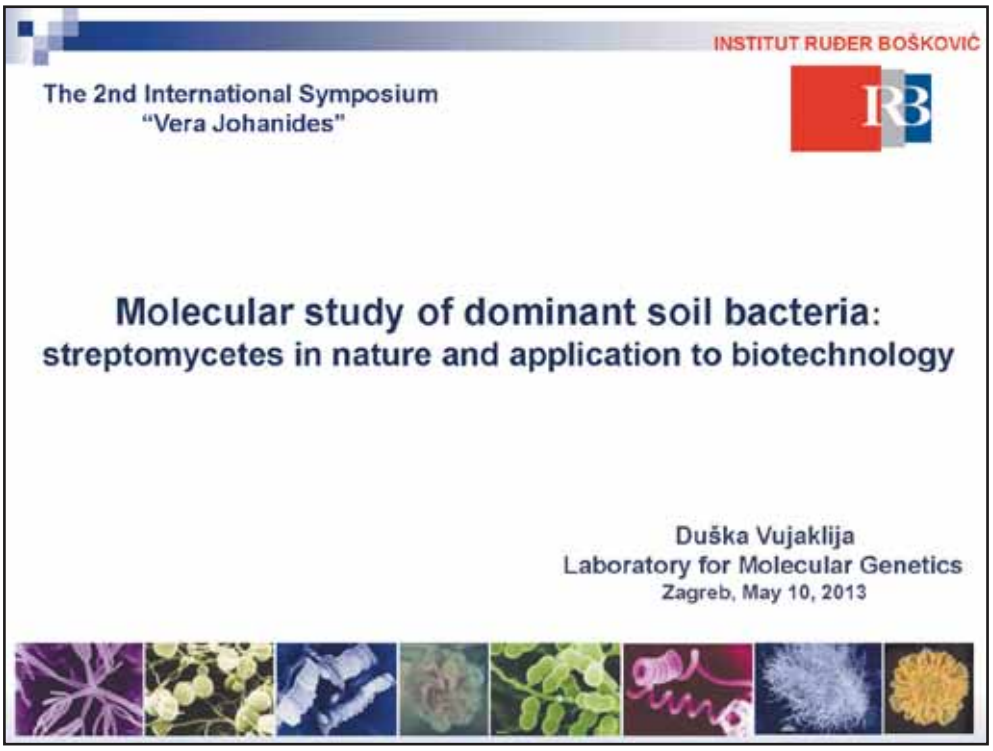

RESULTS
Sensory analysis (SA)
SA – SD
SA – total mark + SD
colour
purity of taste
full body
tingling feeling
quality of bitterness


CONCLUSIONS
GENERAL: Gluten-free beer-like beverages from malted buckwheat and quinoa have very good commercial potential as a healthy functional drinks
DRAWBACKS:
The use of commercial enzyme preparations
High cost of grains

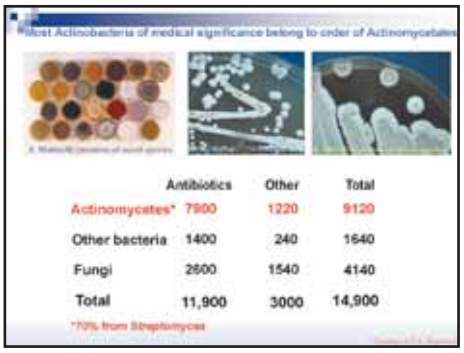

FURTHER WORK
the use of different yeast strains
the impact of successive exploitation of a single starter culture on beverage and yeast properties
breeders and growers should focus on grains with higher enzymatic content
promotion and rising awarnes about pseudocereals benefits

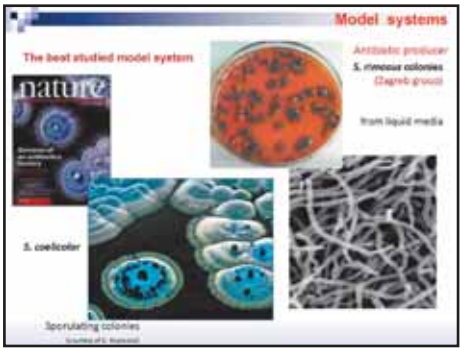

THIS WORK WAS SUPPORTED BY...
Slovenian Research Agency
SLOVENE HUMAN RESOURCES DEVELOPMENT AND SCHOLARSHIP FUND

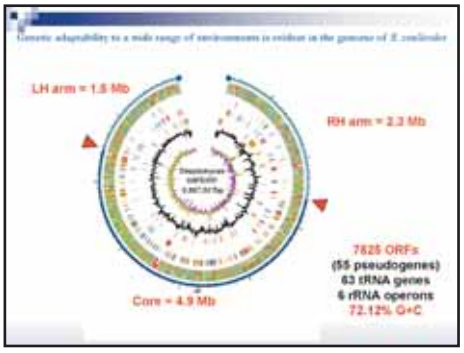

THANK YOU...
...FOR YOUR ATTENTION!

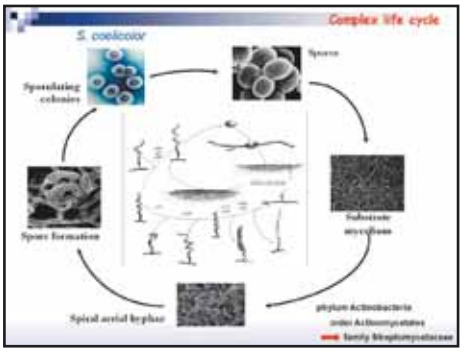

INSTITUT RUĐER BOŠKOVIĆ
The 2nd International Symposium
"Vera Johanides"
Molecular study of dominant soil bacteria:
streptomycetes in nature and application to biotechnology
Duška Vujaklija
Laboratory for Molecular Genetics
Zagreb, May 10, 2013


Actinobacteria - one of the major communities of the microbial population present in soil
• inhabit a wide range of environmental niches; soil, freshwater, marine sediments
• Gram-positive bacteria
• produce a number of enzymes that help degrade organic plant material, lignin, and chitin...
•the best known known as secondary metabolite producers; Streptomyces as antibiotic producers

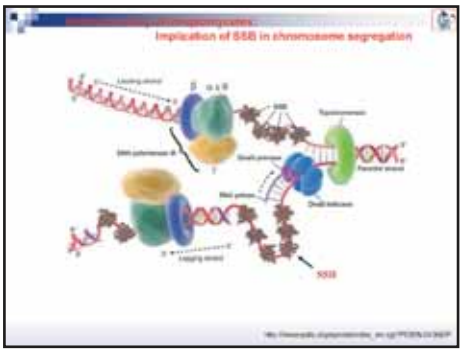

Most Actinobacteria of medical significance belong to order of Actinomycetales
Antibiotics Other Total
Actinomycetes* 7900 1220 9120
Other bacteria 1400 240 1640
Fungi 2600 1540 4140
Total 11,900 3000 14,900
*70% from Streptomyces

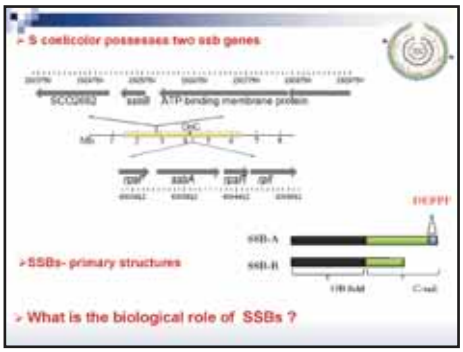

Model systems
The best studied model system
nature
S. coelicolor
Antibiotic producer
S. rimosus colonies
(Zagreb group)
from liquid media
Sporulating colonies

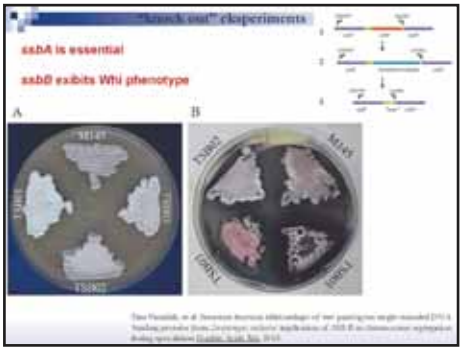

LH arm = 1.5 Mb
RH arm = 2.3 Mb
Core = 4.9 Mb
7825 ORFs
(55 pseudogenes)
63 tRNA genes
6 rRNA operons
72.12% G+C

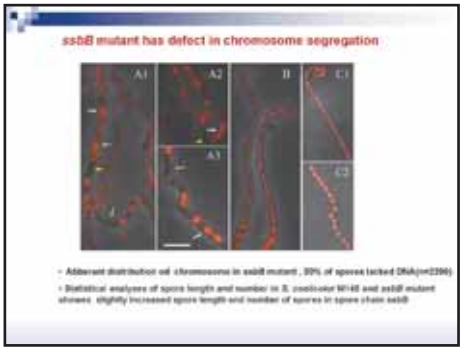

Complex life cycle
S. coelicolor
Sporulating colonies
Spores
Spore formation
Substrate mycelium
Spiral aerial hyphae
phylum Actinobacteria
order Actinomycetales
family Streptomycetaceae

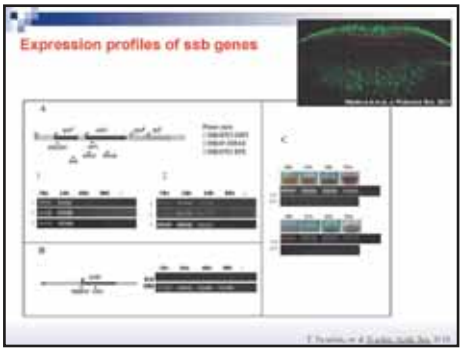

Reproductive stage of S. coelicolor growth
20μm


Implication of SSB in chromosome segregation
SSB

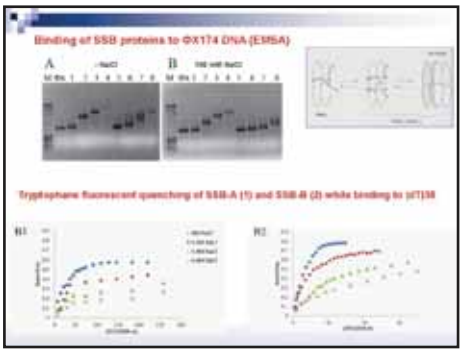

S coelicolor possesses two ssb genes
SCO2462
ssbB
ATP binding membrane protein
ssbA
rpsF
rpl
SSB-A
SSB-B
SSBs- primary structures
What is the biological role of SSBs ?

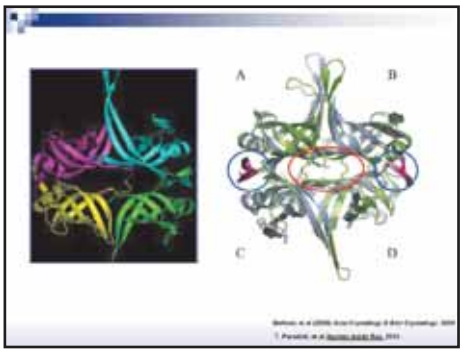

"knock out" eksperiments
ssbA is essential
ssbB exibits Whi phenotype
A
B

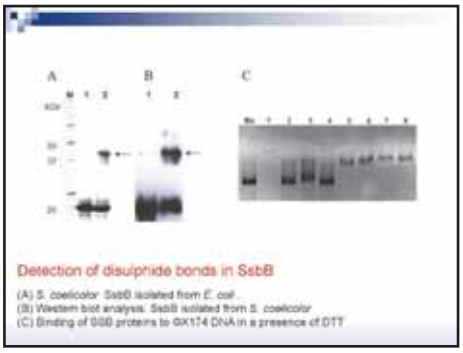

ssbB mutant has defect in chromosome segregation
A1
A2
A3
B
C1
C2

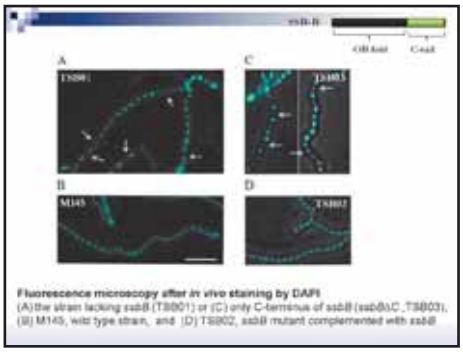

Expression profiles of ssb genes

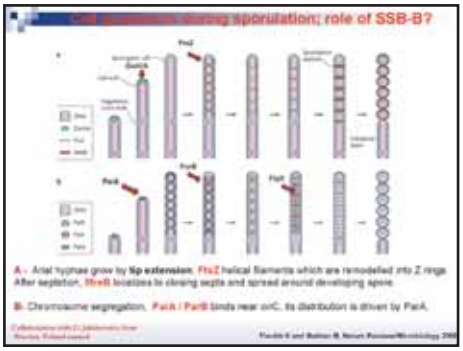

Promoter regions of two ssb genes


Binding of SSB proteins to ΦX174 DNA (EMSA)
Tryptophane fluorescent quenching of SSB-A (1) and SSB-B (2) while binding to (dT)38

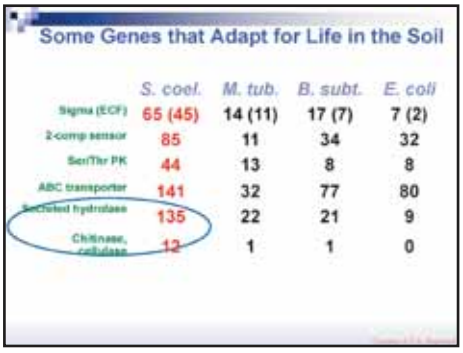

A
B
C
D


Detection of disulphide bonds in SsbB
(A) S. coelicolor SsbB isolated from E. coli .
(B) Western blot analysis. SsbB isolated from S. coelicolor
(C) Binding of SSB proteins to ΦX174 DNA in a presence of DTT

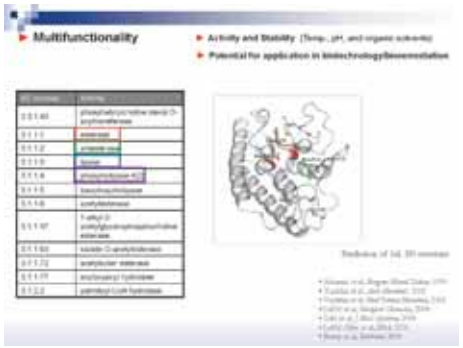

Fluorescence microscopy after in vivo staining by DAPI
(A) the strain lacking ssbB (TS801) or (C) only C-terminus of ssbB (ssbBΔC ,TS803),
(B) M145, wild type strain, and (D) TS802, ssbB mutant complemented with ssbB


A - Aerial hyphae grow by tip extension. FtsZ helical filaments which are remodelled into Z rings.
After septation, MreB localizes to closing septa and spread around developing spore.
B- Chromosome segregation. ParA / ParB binds near oriC, its distribution is driven by ParA.

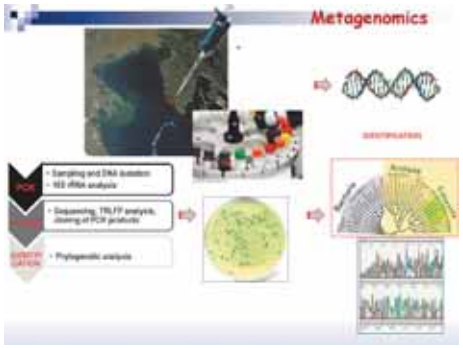

Streptomyces: still represent an excellent source for genome mining


Some Genes that Adapt for Life in the Soil
S. coel. M. tub. B. subt. E. coli
Sigma (ECF) 65 (45) 14 (11) 17 (7) 7 (2)
2-comp sensor 85 11 34 32
Ser/Thr PK 44 13 8 8
ABC transporter 141 32 77 80
Secreted hydrolase 135 22 21 9
Chitinase, cellulase 12 1 1 0


Genome mining
CDSL lipolytic family


Multifunctionality
Activity and Stability (Temp., pH, and organic solvents)
Potential for application in biotechnology/bioremediation


Taxonomic distribution
Scanning for genes encoding GDS(L) hydrolases in Actinobacteria from wide diversity of ecological niches
Ana Bielen
The 2nd International Symposium "VERA JOHANIDES", 2013
Zagreb, May 11, 10.40 am


Metagenomics
PCR
Sampling and DNA isolation
16S rRNA analysis
Sequencing, TRLFP analysis, cloning of PCR products
Phylogenetic analysis


Ana Pantaleta, IRB
University of Strathclyde, Glasgow

The 2nd International Symposium "Vera Johanides" -
BIOTECHNOLOGY IN CROATIA BY 2020
Zagreb, May 10-11, 2013

CROATIAN ACADEMY OF ENGINEERING

MOLECULAR GENETICS: THE PRESENT AND THE FUTURE

Prof. Dragan Primorac, M.D.,Ph.D.

Eberly College of Science, Penn State University, USA,
University of New Haven, USA,
University of Split, School of Medicine, Medicinski fakultet,
University of Osijek, School of Medicine, Osijek

E-mail: draganprimorac2@gmail.com
Web: draganprimorac.com
draganprimorac.org

Human genome

- Humans are estimated to have **23,686 genes**.
- Only about **1.5-3 %** of the genome codes proteins.
- Even less number of genes can produce proteins.
- One gene can produce more than one protein.
- Rest consists of non-coding RNA, regulatory sequence, introns, noncoding (junk DNA)

Human genome

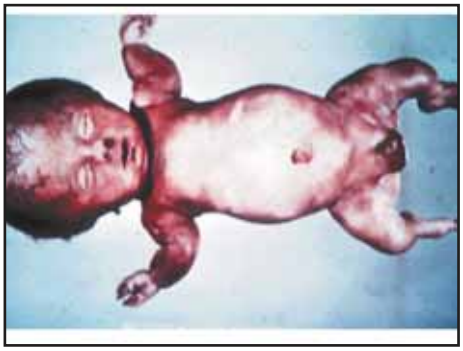
- Human cells have **23 pairs chromosomes** (22 pairs of autosomes and one pair of sex chromosomes), giving a total of 46 per cell
- Humans are diploid (We have **two copies of our genome in each somatic cells**. Only exception is mature red blood cells which become enucleated during development and therefore lack a genome)
- The human haploid genome (one copy of the genome) is **3.2 Gb** (3.2 billion bp)
- **99,9 %** of all humans are having identical DNA

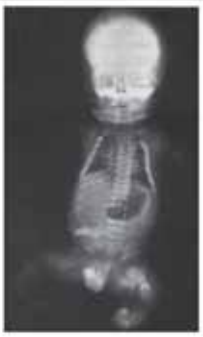
An organism's complexity is not directly proportional to its genome size, number of genes or number of chromosomes

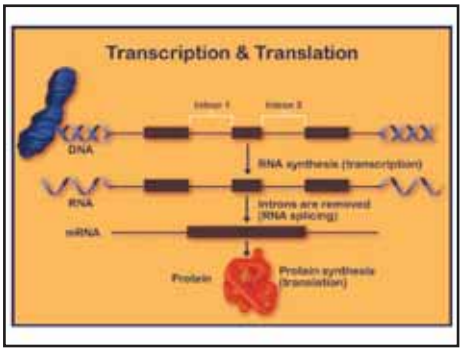
The **Human haploid genome** (one copy of the genome) is 3.2 Gb (3.2 billion bp) distributed 23 chromosome while the haploid genome of **Mouse genome** is about 3.0 Gb (3.0 billion bp) distributed over 20 chromosome. The current estimated gene count in mouse genome is 23,786 while in human is 23,688.

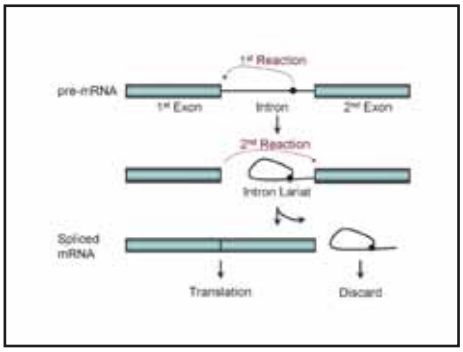
- ***Polychaos dubium*** (freshwater amoeboid) have largest genome known consisting 670 billion base pairs (200 times larger than human genome)

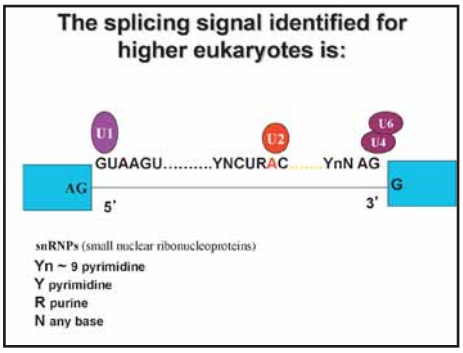
Molecular medicine today

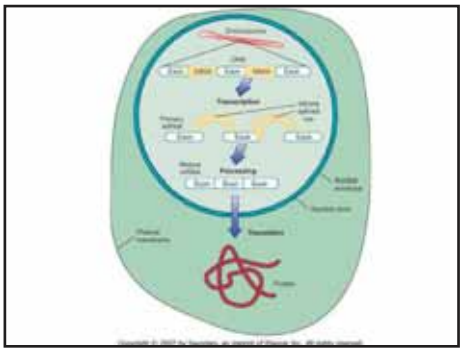




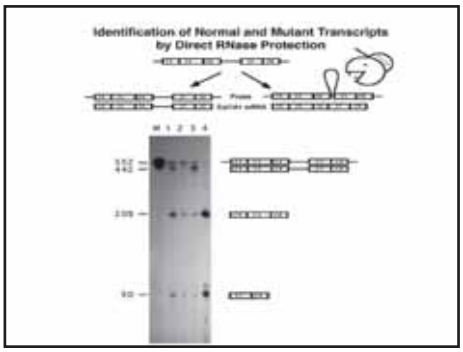

Transcription & Translation
DNA
RNA synthesis (transcription)
RNA
Introns are removed (RNA splicing)
mRNA
Protein
Protein synthesis (translation)

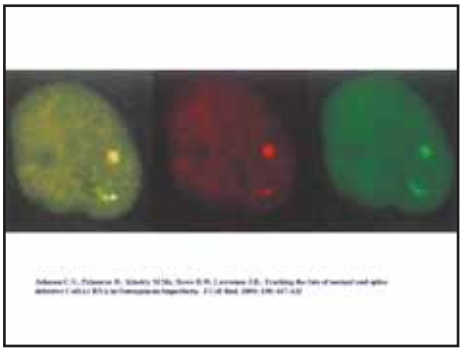

1st Reaction
pre-mRNA
1st Exon
Intron
2nd Exon
2nd Reaction
Intron Lariat
Spliced mRNA
Translation
Discard

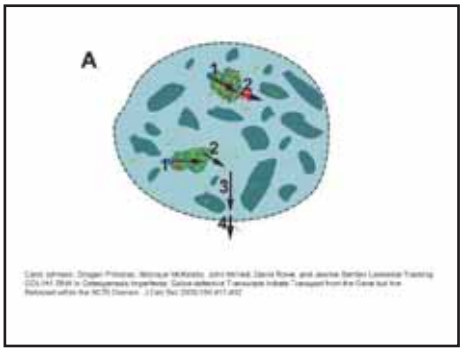

The splicing signal identified for higher eukaryotes is:
U1
U2
U6
U4
GUAAGU.....YNCURAC.....YnN AG
AG
G
5'
3'
snRNPs (small nuclear ribonucleoproteins)
Yn ~ 9 pyrimidine
Y pyrimidine
R purine
N any base

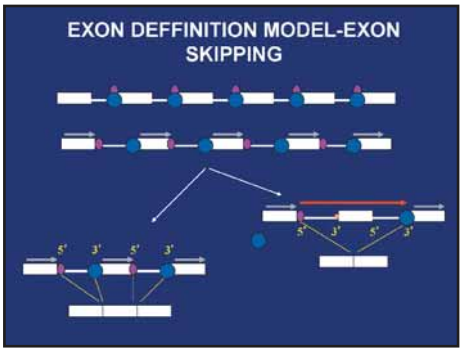


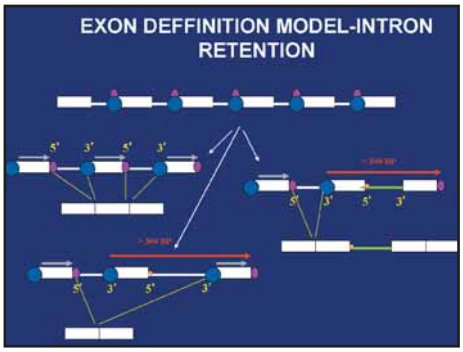

SEQUENCING
G A T C G A T C
GTC gtaagtatct
GTC ataagtatct

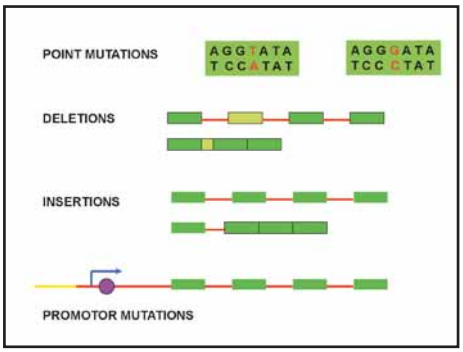

Identification of Normal and Mutant Transcripts by Direct RNase Protection

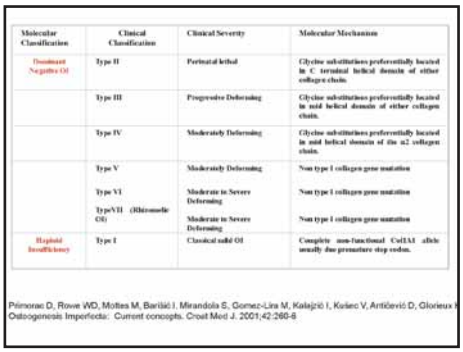



A



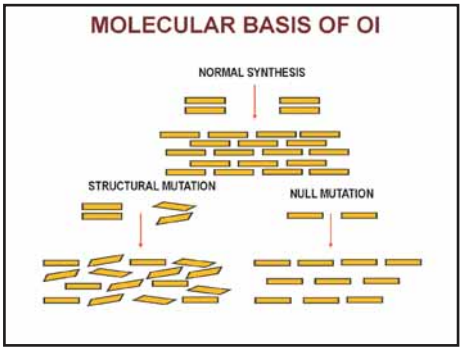


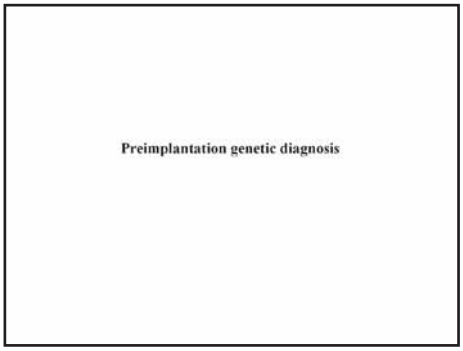


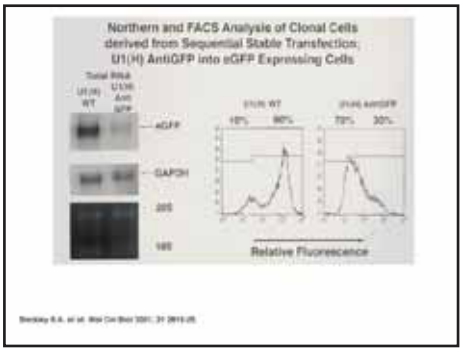


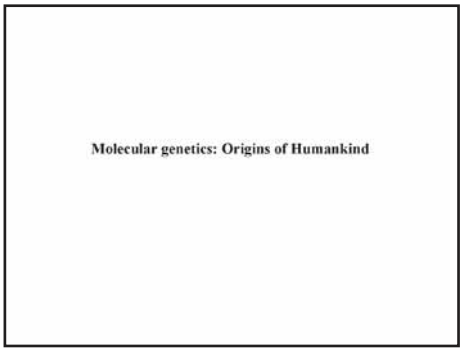
Molecular Classification	Clinical Classification	Clinical Severity	Molecular Mechanism
Dominant Negative OI	Type II	Perinatal lethal	Glycine substitutions preferentially located in C terminal helical domain of either collagen chain.
	Type III	Progressive Deforming	Glycine substitutions preferentially located in mid helical domain of either collagen chain.
	Type IV	Moderately Deforming	Glycine substitutions preferentially located in mid helical domain of the α2 collagen chain.
	Type V	Moderately Deforming	Non type I collagen gene mutation
	Type VI	Moderate to Severe Deforming	Non type I collagen gene mutation
	TypeVII (Rhizomelic OI)	Moderate to Severe Deforming	Non type I collagen gene mutation
Haploid Insufficiency	Type I	Classical mild OI	Complete non-functional Col1A1 allele usually due premature stop codon.

Primorac D, Rowe WD, Mottes M, Barišić I, Mirandola S, Gomez-Lira M, Kalajzić I, Kušec V, Antičević D, Glorieux F
Osteogenesis Imperfecta: Current concepts. Croat Med J. 2001;42:260-8

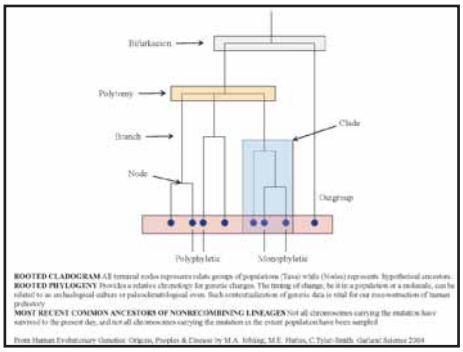




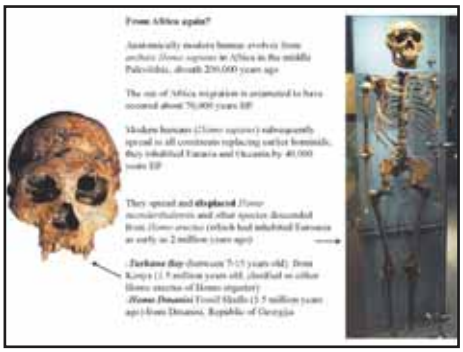


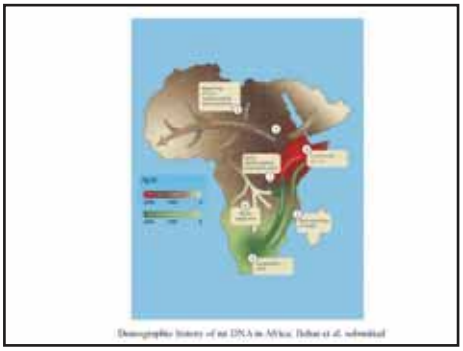



TWO CRITICAL QUESTIONS:
Where did we come from?
What makes us human?


Bifurkasen
Polytomy
Branch
Node
Clade
Outgroup
Polyphyletic
Monophyletic

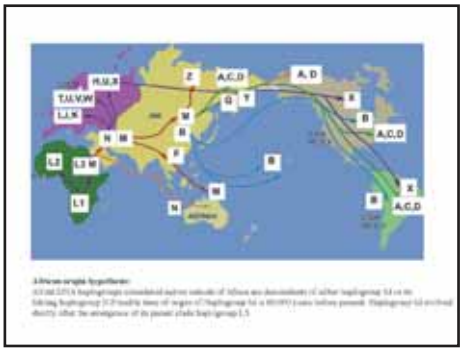

AFRICAN EXODUS

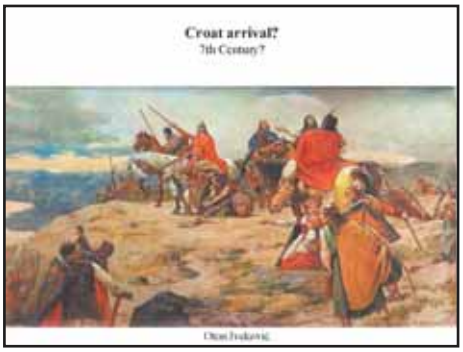

From Africa again?



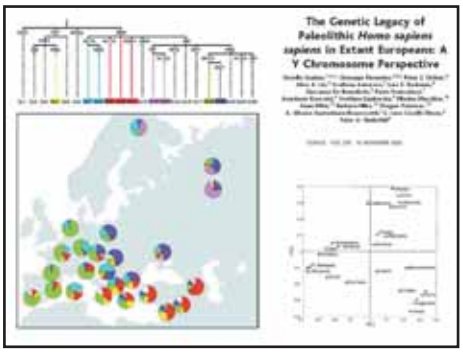

L3
L2
L4
M1
L1
L5
L
Eve-m
L0
200-130 Kya
130 - 90 Kya
90 - 55 Kya
L0d
L0k

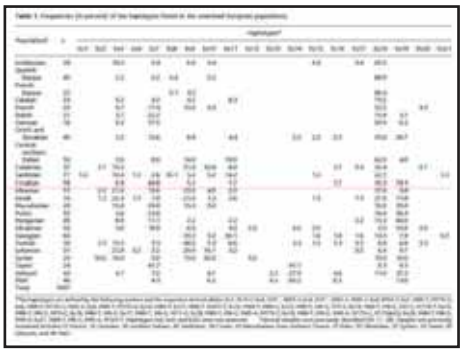

Red Sea

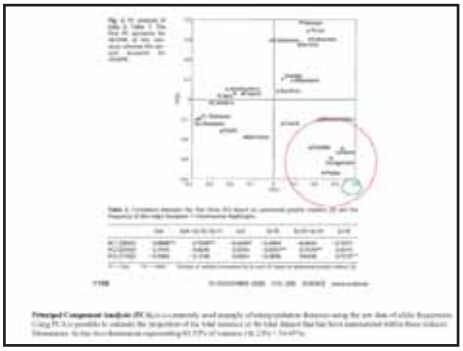

A,C,D
A, D
L1
L2
L3 M
A,C,D


Croat arrival?
7th Century?

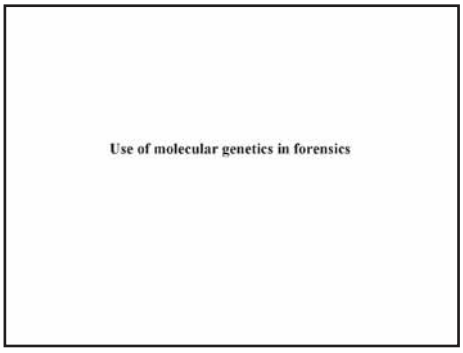

Anthropology in our lab (Split)

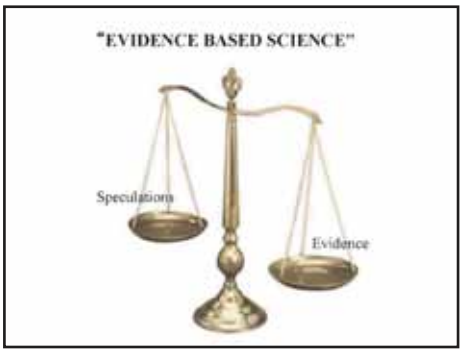

The Genetic Legacy of Paleolithic Homo sapiens sapiens in Extant Europeans: A Y Chromosome Perspective

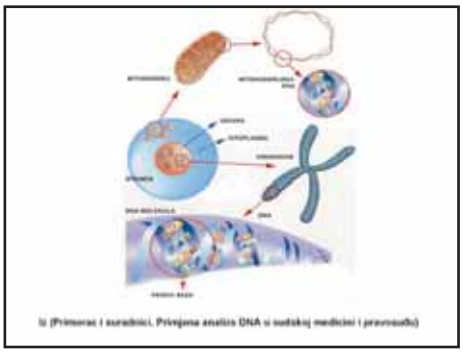




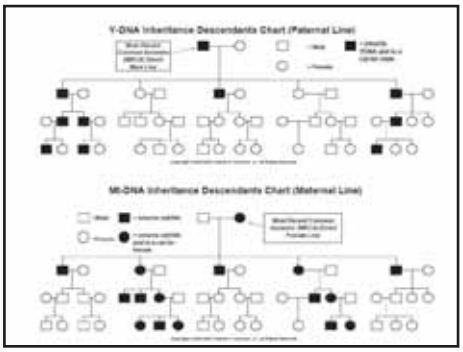

Science

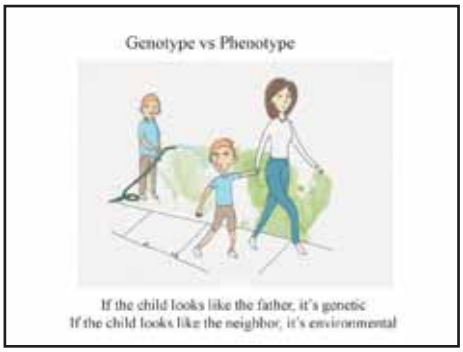

Use of molecular genetics in forensics

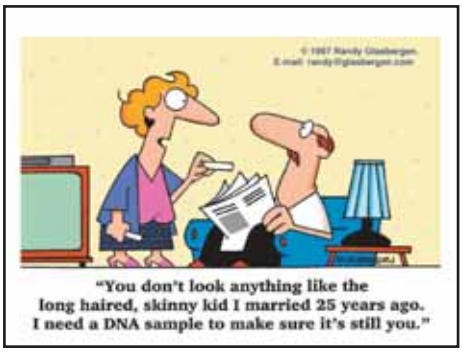




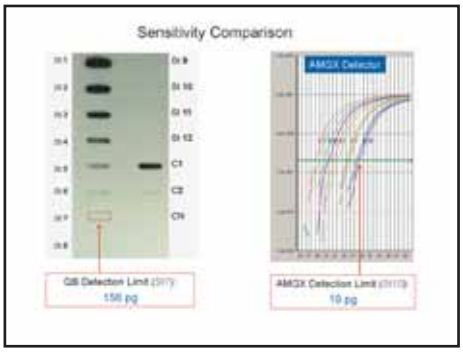
Iz (Primorac i suradnici, Privijena analiza DNA u sudskoj medicini i pravosuđu)

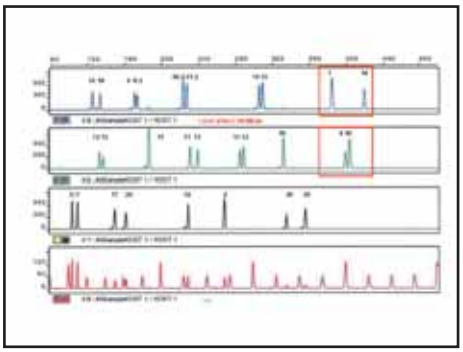


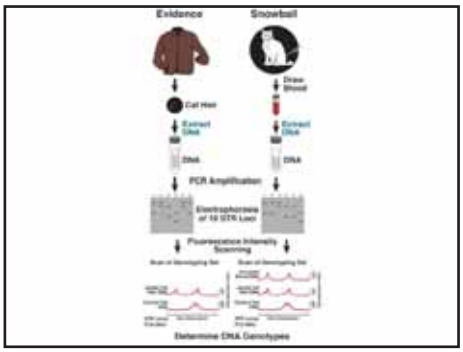




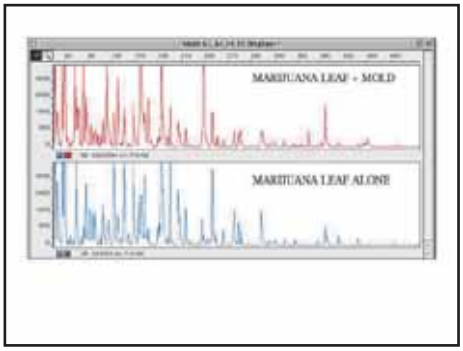

57/96


Sensitivity Comparison
AMGX Detector
QS Detection Limit
150 pg
AMGX Detection Limit
10 pg

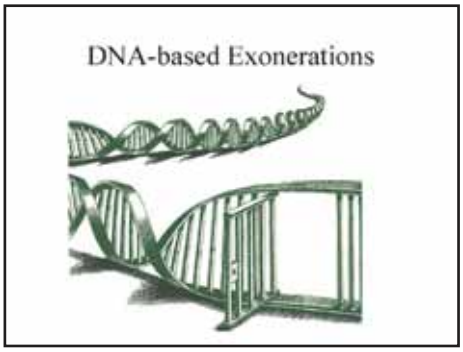



Evidence
Snowball
PCR Amplification

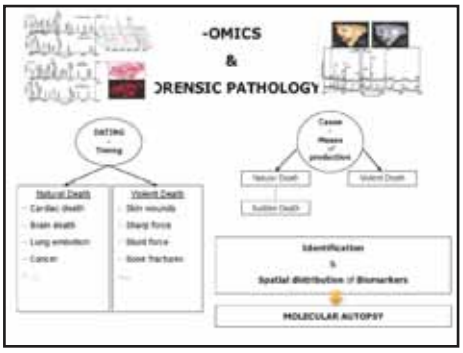

CONSTRUCTION OF A NATIONWIDE MARIJUANA AFLP DATABASE
HOW MUCH IS CLONAL?
CAN WE DISTINGUISH AMONG VARIETIES?
CAN WE LINK GROW OPERATIONS TOGETHE
CAN WE LINK DISTRIBUTORS?

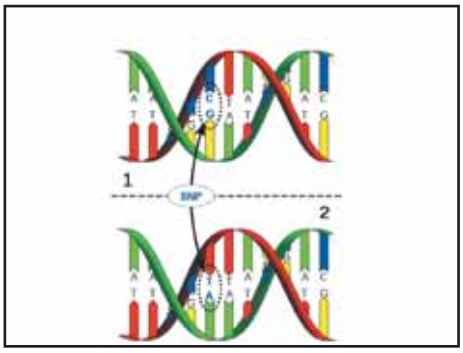

MARIJUANA LEAF + MOLD
MARIJUANA LEAF ALONE

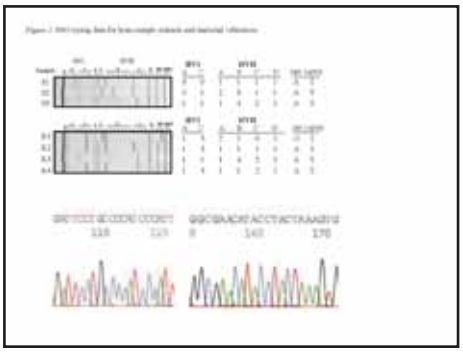

Over 190 seizure cases
CANADA
Pacific Ocean
Ocean
MEXICO

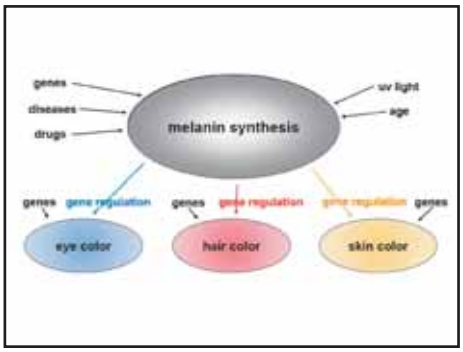

DNA-based Exonerations

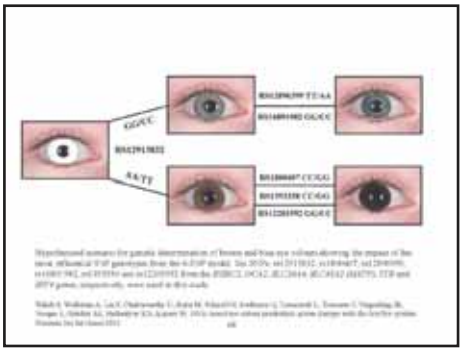

FORENSIC MOLECULAR BIOLOGY TODAY
RECENT ADVANCES IN FORENSIC MOLECULAR BIOLOGY
STR, mtDNA, Y and X-chromosome
PCR


-OMICS
&
ORENSIC PATHOLOGY
DATING
Timing
Cause
Means
of production
Natural Death
Violent Death
Identification
&
Spatial distribution of Biomarkers
MOLECULAR AUTOPSY


1
SNP
2




genes
diseases
drugs
melanin synthesis
uv light
age
genes
gene regulation
genes
gene regulation
gene regulation
genes
eye color
hair color
skin color



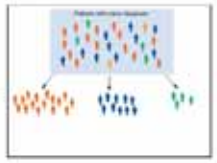
The future of the molecular medicine

Individualized/Personalized Medicine: Dream or Reality?

Personalised medicine

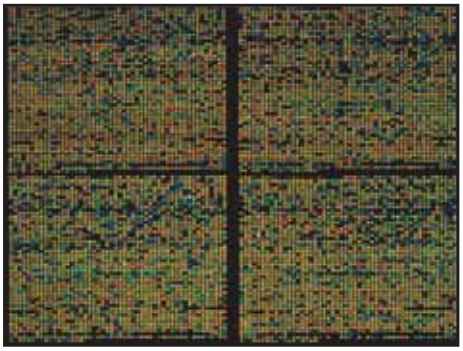
Clinical view

- Pharmacogenetics (study of inherited variation in drug response)
- Aim in the medical society
  - "Right drug to the right patient at a right time with right dosage"
  - individually tailored drug treatment / therapy
    - more efficient medication
    - less therapeutic failures and adverse drug reactions



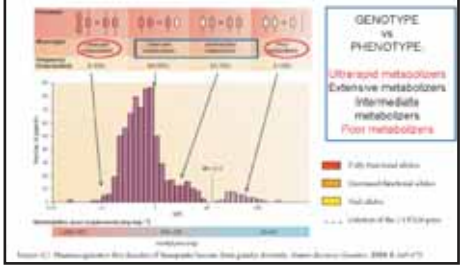
Practice of medicine tomorrow

- Personalized or individualized medicine
  - Prevent disease
  - Apply therapy appropriate to the patient
  - Screen family members for risk
  - Prevent disease in family/ cure disease



Pharmacogenetics

- Genotypes vs. phenotypes



PREDICTED METABOLIZER PHENOTYPE (RANGE MULTI-ETHNIC FREQUENCY)

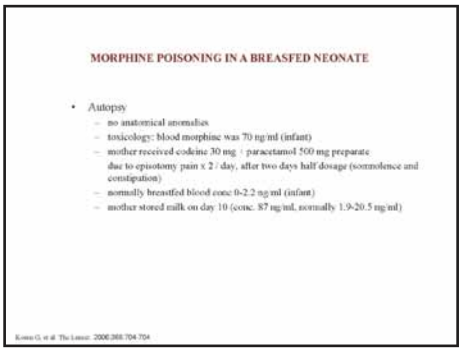
Clinical Pharmacogenetics Implementation Consortium (CPIC) guidelines for cytochrome P450 2D6 (CYP2D6) genotype and codeine therapy

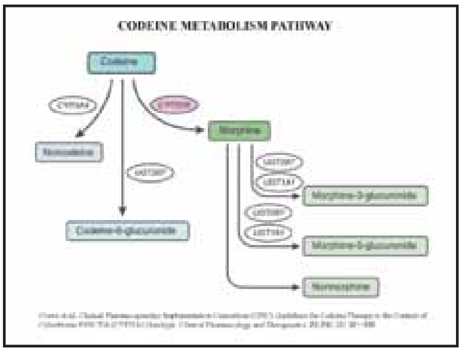
Allele	*1	*2	*1xN or *2xN	*3	*4 or *4xN	*5	*6	*10	*17	*41
*1	EM	EM	EM	EM	EM	EM	EM	EM	EM	EM
*2		EM	UM	EM	EM	EM	EM	EM	EM	EM
*1xN or *2xN			UM	EM or UM	EM or UM	EM or UM	EM or UM	UM	UM	UM
*3				PM	PM	PM	PM	IM	IM	IM
*4					PM	PM	PM	IM	IM	IM
*5						PM	PM	IM	IM	IM
*6							PM	IM	IM	IM
*10								EM	EM	EM
*17									EM	EM
*17										EM

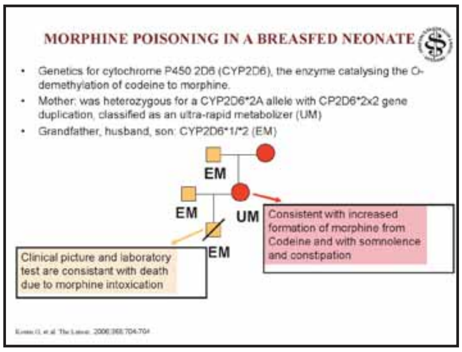
EM: extensive metabolizer; IM: intermediate metabolizer; PM: poor metabolizer; UM: ultrarapid metabolizer

Is It Safe to Use Opioids for Obstetric Pain while Breastfeeding?

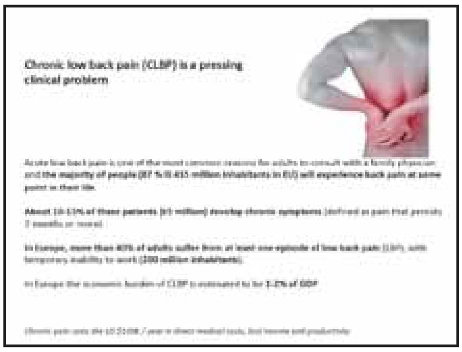

A CASE REPORT: MORPHINE POISONING IN A BREASTFED NEONATE
A full-term healthy male infant
Infant was showing intermittent difficulties in breasfeeding
Lethargy on day 7
Grey skin and milk intake decrease on day 12
Found dead on day 13

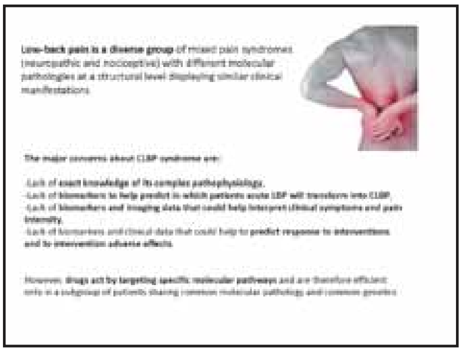

MORPHINE POISONING IN A BREASFED NEONATE
Autopsy
no anatomical anomalies
toxicology: blood morphine was 70 ng/ml (infant)
mother received codeine 30 mg + paracetamol 500 mg preparate due to episotomy pain x 2 / day, after two days half dosage (somnolence and constipation)
normally breastfed blood conc 0-2.2 ng/ml (infant)
mother stored milk on day 10 (conc. 87 ng/ml, normally 1.9-20.5 ng/ml)


CODEINE METABOLISM PATHWAY
Codeine
Morphine
Norcodeine
Codeine-6-glucuronide
Morphine-3-glucuronide
Morphine-6-glucuronide
Normorphine

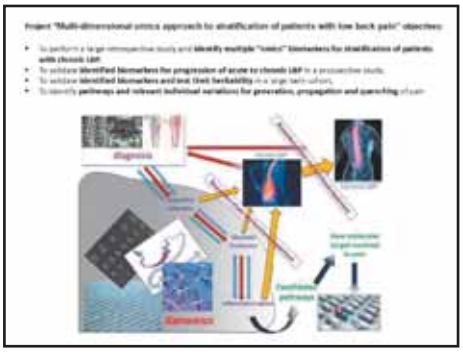

MORPHINE POISONING IN A BREASFED NEONATE
Genetics for cytochrome P450 2D6 (CYP2D6), the enzyme catalysing the O-demethylation of codeine to morphine.
Mother: was heterozygous for a CYP2D6*2A allele with CP2D6*2x2 gene duplication, classified as an ultra-rapid metabolizer (UM)
Grandfather, husband, son: CYP2D6*1/*2 (EM)
EM
EM
UM
EM
Consistent with increased formation of morphine from Codeine and with somnolence and constipation
Clinical picture and laboratory test are consistant with death due to morphine intoxication


Multi-dimensional omics approach to stratification of patients with low back pain


Chronic low back pain (CLBP) is a pressing clinical problem


Low-back pain is a diverse group of mixed pain syndromes (neuropathic and nociceptive) with different molecular pathologies at a structural level displaying similar clinical manifestations
The major concerns about CLBP syndrome are:


St. Catherine
GENOS
Our clinical centres are treating over 4,000 new patients with CLBP each year.



Replacement Organs and Tissue?

ISABS Conference on Forensic, Anthropologic and Medical Genetics and Mayo Clinic Lectures in Translational Medicine

The most important scientific event in Europe during 2013.

Conference is organized by International Society for Applied Biological Sciences and co-organized by **Mayo Clinic** (USA), **Penn State University** (USA), **University of New Haven** (USA), **George Washington University** (USA), Institute Ruđer Bošković, Universities of Split, Rijeka and Osijek etc.

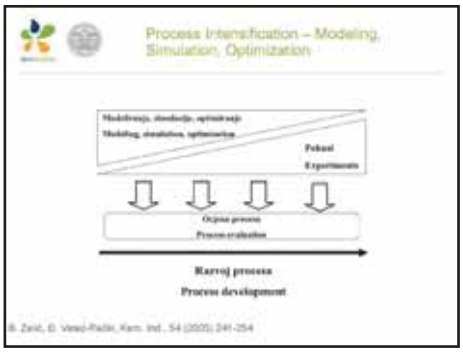
The 8th ISABS conference will feature some 80 distinguished lecturers from the organizing institutions and other scientific research centers (**Harvard School of Medicine, Stanford University, Armed Forces Institute of Pathology, Baylor College of Medicine, National Institute of Health, Cleveland Clinic, Max Planck Institute, George Washington University, The Weizmann Institute of Science** etc).

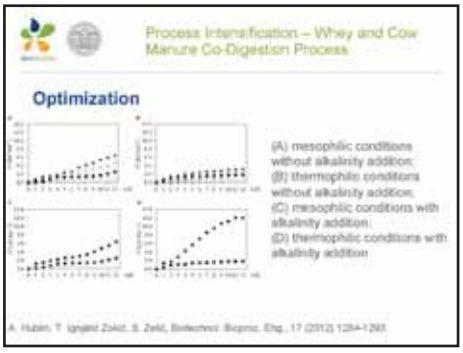
Nobel Prize laureates **Aaron Ciechanover, Robert Huber and Ada Yonath** key note lecturers.

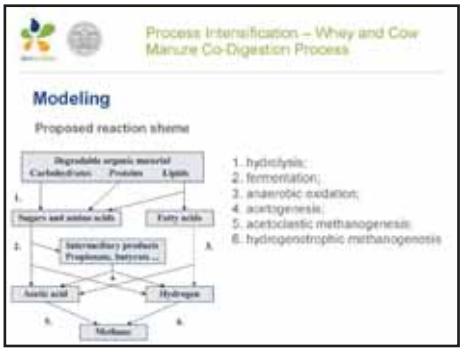
500 participants from more than 40 countries.

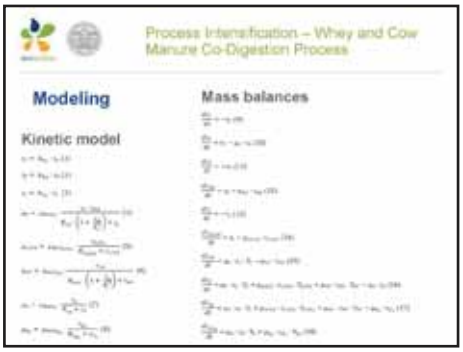

Sveučilište u Zagrebu
Fakultet kemijskog inženjerstva i tehnologije
Intensification of Agro and Food Industry Waste Biodegradation Process
Marina Tišma, Natalija Velić, Mario Panjičko, Bruno Zelić

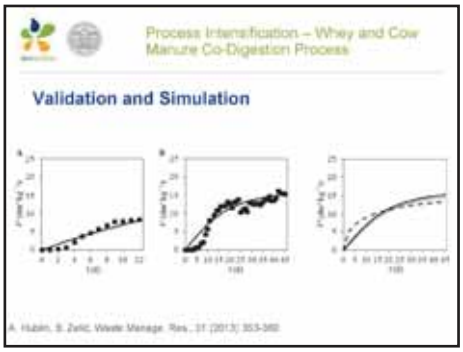

Outlook
Process Intensification
- Modeling, Simulation and Optimization
- Waste Preatretment
- Scale-up – Mobile Pilot Plant

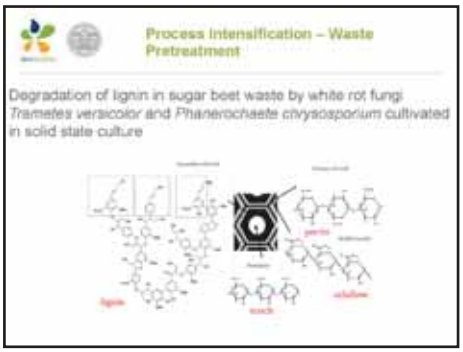

Process Intensification – Modeling, Simulation, Optimization
Razvoj procesa
Process development


Process Intensification – Whey and Cow Manure Co-Digestion Process
Optimization
(A) mesophilic conditions without alkalinity addition;
(B) thermophilic conditions without alkalinity addition;
(C) mesophilic conditions with alkalinity addition;
(D) thermophilic conditions with alkalinity addition

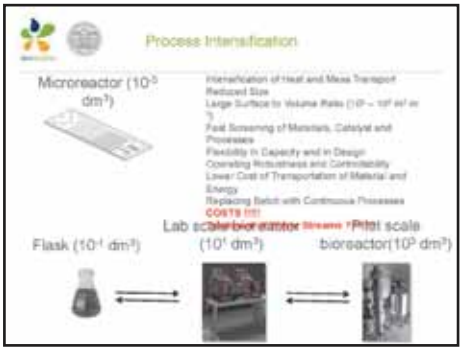

Process Intensification – Whey and Cow Manure Co-Digestion Process
Modeling
Proposed reaction sheme
1. hydrolysis;
2. fermentation;
3. anaerobic oxidation;
4. acetogenesis;
5. acetoclastic methanogenesis;
6. hydrogenotrophic methanogenesis

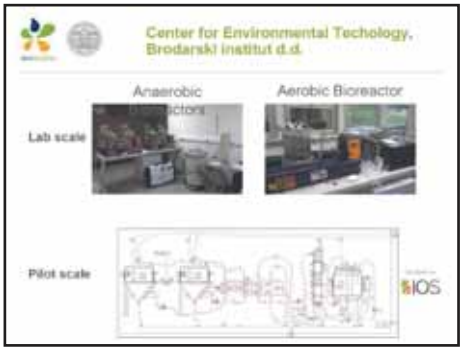

Process Intensification – Whey and Cow Manure Co-Digestion Process
Modeling
Kinetic model
Mass balances


Process Intensification – Whey and Cow Manure Co-Digestion Process
Validation and Simulation


Process Intensification – Waste Pretreatment
Degradation of lignin in sugar beet waste by white rot fungi *Trametes versicolor* and *Phanerochaete chrysosporium* cultivated in solid state culture


Process Intensification – Waste Pretreatment
Sugar beet waste degradation after 30 days of solid state fermentation
P. chrysosporium
T. versicolor
C : N = 36.8 : 1
t = 0 day

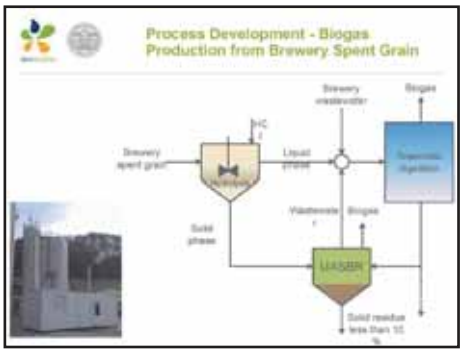

Process Intensification
Microreactor (10⁻⁵ dm³)
Flask (10⁻¹ dm³)
Lab scale (10¹ dm³)
Pilot scale bioreactor(10³ dm³)


Center for Environmental Techology, Brodarski institut d.d.
Anaerobic
Aerobic Bioreactor
Lab scale
Pilot scale


Center for Environmental Techology, Brodarski institut d.d.
Anaerobic
Aerobic Bioreactor
Lab scale
Mobile Pilot Plant


Mobile Pilot Plant
Two Solid State Reactors
- solid waste
- a(na)erobic conditions
- V = 200 dm³
Anaerobic Reactor
- liquid waste
- a(na)erobic treatment of wastewaters
- stirring and pH regulation
UASB Reactor
- Upflow Anaerobic Sludge Blanket Reactor
- anaerobic treatment of sludge samples
- V = 40 dm³


Biogas Production from Brewery Spent Grain
Brewery Laško
Capacity: 100,000,000 L of brew annually
Project: treatment of brewery waste streams
Wastewater – done
Yeast – done
Spent grain – development in progress
Brewery spent grain:
• lignocellulosic material containing about 17 % cellulose, 28 % non-cellulosic polysaccharides, mostly arabinoxylans, and 28 % lignin
• 0.8-1.2 m³/kg dry organic matter → 120-130 m³ biogas/ton
• total usable biogas potential
biogas 1,000,000-2,000,000 m³


Process Development - Biogas Production from Brewery Spent Grain
Brewery wastewater
Biogas
Brewery spent grain
Liquid phase
Solid phase
Biogas
Solid residue less than 10 %


Acknowledgment
hrzz
PTE
BICRO

RHEOLOGY OF LIQUID MEDIA INFLUENCES BACTERIAL PHYSIOLOGY

Maja Borić, Tjaša Danevčič, David Stopar

University of Ljubljana, Biotechnical Faculty

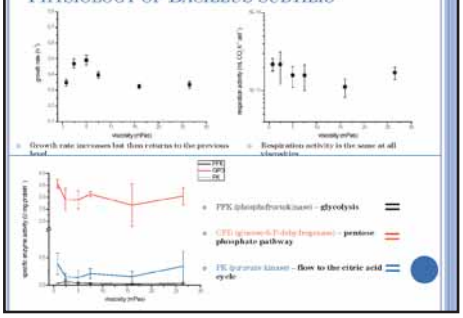
IMPORTANCE OF RHEOLOGY IN BIOTECHNOLOGICAL PROCESSES

- Rheology studies the flow of liquids or soft matter
- Water treatment plants, bioreactors
- Influences pumping, hydrodynamics, mass transfer rates and sludge-water separation
- Changes molecular diffusion as well as movement of microorganisms
- **Can a change in liquid medium rheology cause a change in bacterial physiology?**

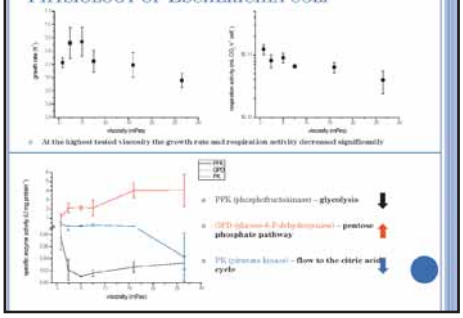
CHANGING MEDIUM RHEOLOGY IN THE LAB

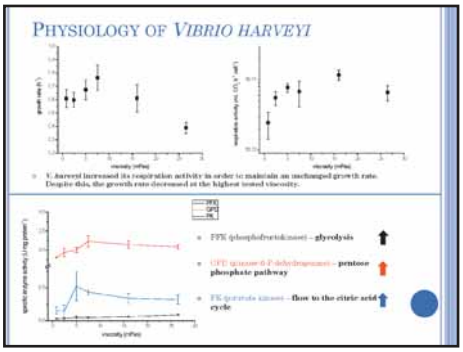
- Ideal polymer:
  - increases viscosity
  - changes Newtonian liquid to non-Newtonian
  - can not be degraded by bacteria and used as a nutrient
  - does not affect other physico-chemical factors (pH, water activity, DOC)
- **Hydroxyethyl cellulose (HEC)**
- Viscosity range: 0.8 mPas – 26.5 mPas
- Model organisms:
  - *Bacillus subtilis*
  - *Escherichia coli*
  - *Vibrio harveyi*

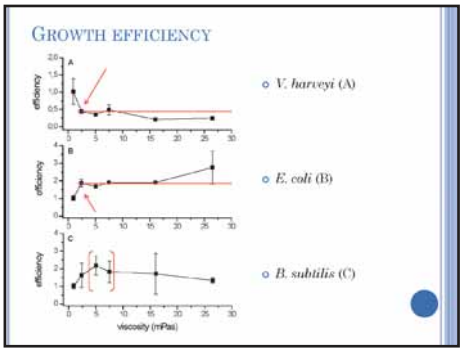
PHYSIOLOGY OF *BACILLUS SUBTILIS*

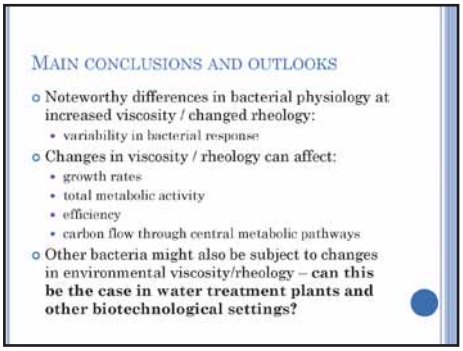


PHYSIOLOGY OF *ESCHERICHIA COLI*




PHYSIOLOGY OF VIBRIO HARVEYI
PFK (phosphofructokinase) – glycolysis
pentose phosphate pathway
flow to the citric acid cycle


GROWTH EFFICIENCY
efficiency
viscosity (mPas)
V. harveyi (A)
E. coli (B)
B. subtilis (C)


MAIN CONCLUSIONS AND OUTLOOKS
Noteworthy differences in bacterial physiology at increased viscosity / changed rheology:
variability in bacterial response
Changes in viscosity / rheology can affect:
growth rates
total metabolic activity
efficiency
carbon flow through central metabolic pathways
Other bacteria might also be subject to changes in environmental viscosity/rheology – can this be the case in water treatment plants and other biotechnological settings?


ACKNOWLEDGEMENTS
co-workers at the Chair of Microbiology, Biotechnical Faculty, Ljubljana
prof. dr. David Stopar
dr. Tjaša Danevčič
JUB, d.d.
JUB
Slovenian Research Agency


Green roofs -
future of urban life
Jure Šumi
KNAUF INSULATION
urbanscape
Above and beyond


Green Roofs


Types of green roofs


Why green roofs
Water
Cost
Air
Social benefits


Main positive effects of green roofs


Main positive effects of green roofs
Significant impact on CO2 emissions
Cut back on the amount of dust and other pollutants in the air
Better storm water management
Increased Roofing Membrane Durability
Increased Biodiversity
Positive impact on people


Traditional extensive green roof systems
Standard basic components / layers:
Vegetation
Growing media
Filter membrane
Drainage
Root barrier
Waterproofing


Main pitfalls of traditional systems
Variable water absorption capacity
Heavy
Labour intensive
Costly


What to change?
It is all about substrate and its quality & performance!
Good substrate:
Needled rock mineral wool fibres
combined with superabsorbent polymers


Advantages of Urbanscape
extensive green roof system
High and constant water absorption
rate with long term water retention
stability
Lightness
Low labour intensity
Low cost installation


The new world of new
applications to tackle
urbanscape


Urbanscape roof vegetable gardens


Landscaping applications


Simple system


Urbanscape in-house gardens


Q & A
urbanscape

Sustainable Production of Polyhydroxyalkanoates

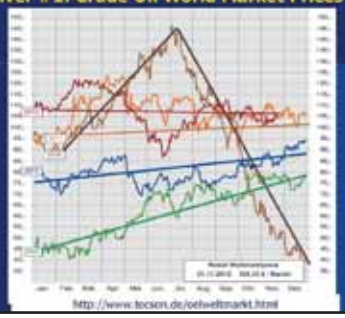
Gerhart Braunegg[1]
Martin Koller[2]

[1] ARENA, c/o TU Graz, 8010 Graz, Inffeldgasse 21 b,
E-mail: g.braunegg@tugraz.at

[2] Institute of Biotechnology and Bioprocess Engineering
Graz University of Technology, 8010 Graz, Petersgasse 12

The 2nd International Symposium "VERA JOHANIDES" - BIOTECHNOLOGY IN CROATIA BY 2020
Zagreb, May 10-11, 2013

Driver #1: Crude Oil World Market Prices



http://www.tecson.de/oelweltmarkt.html

Other Drivers

- Industrial Development towards Sustainable Processes and Products
- Paradigm Change in Raw Materials: From Fossile to Renewable
- Prevention or Lowering Influences on the Environment: e.g. Global warming

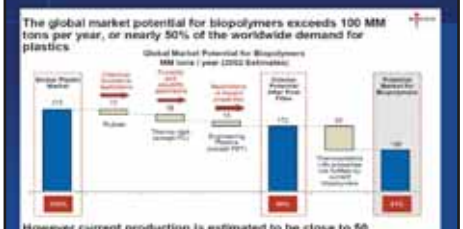
The Consequence

- Production Polymers, Fine Chemicals and Biofuels from Renewable Resources
  - Near Future: Waste and Surplus Materials Generated in Existing Agro-Industrial Processes, e.g. Cheese Whey, Used Oils & Fats, Lignocellulose
  - Later: Agricultural Production of Crops as Raw Materials Algae Biomass ?
- Minimization of Energy Input from Fossil Resources
  - Near Future: Utilization of Lignocellulosic Plant Biomass
  - Later: Photovoltaic, Biohydrogen (?)

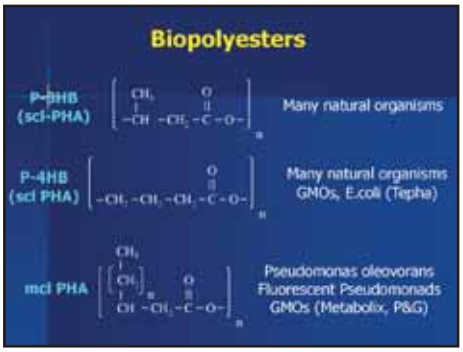
Production via „WHITE BIOTECHNOLOGY"

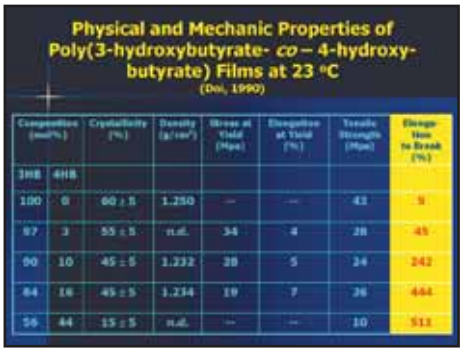
Global Market Potential for Biopolymers

The global market potential for biopolymers exceeds 100 MM tons per year, or nearly 50% of the worldwide demand for plastics

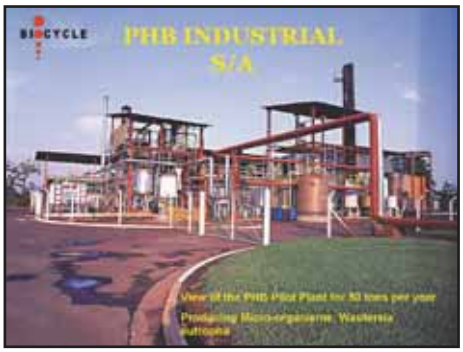


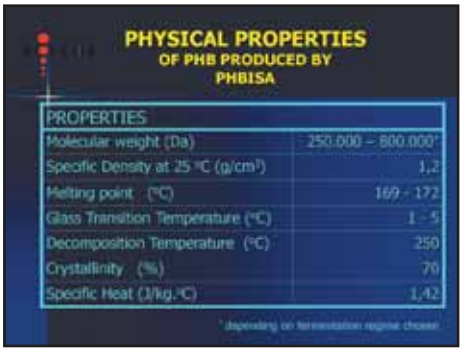
However current production is estimated to be close to 50 thousand tons per year – less than 0.05% of potential

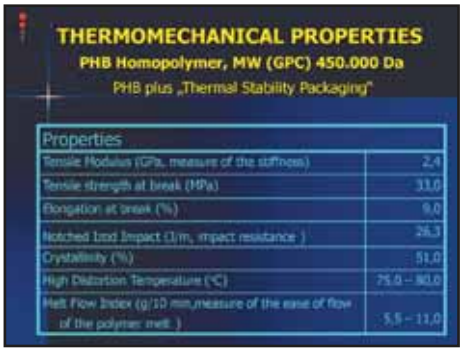

Biopolyesters
P-3HB (scl-PHA)
Many natural organisms
P-4HB (scl PHA)
Many natural organisms GMOs, E.coli (Tepha)
mcl PHA
Pseudomonas oleovorans Fluorescent Pseudomonads GMOs (Metabolix, P&G)

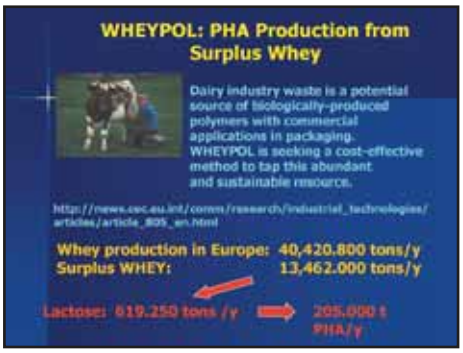

Physical and Mechanic Properties of Poly(3-hydroxybutyrate- co – 4-hydroxy-butyrate) Films at 23 °C
(Doi, 1990)
3HB 4HB
100 0 60 ± 5 1.250 – – 43 5
97 3 55 ± 5 n.d. 34 4 28 45
90 10 45 ± 5 1.232 28 5 24 242
84 16 45 ± 5 1.234 19 7 26 444
56 44 15 ± 5 n.d. – – 10 513


SUGAR CANE AS A RAW MATERIAL
BIO-PRODUCTS
Fine Chemicals
Polymers
Antibiotics
Solvents
Organic Acids
Others

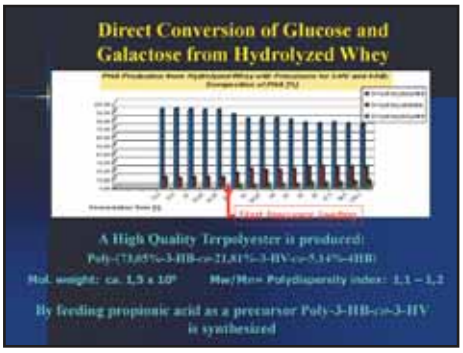

BIOCYCLE
PHB INDUSTRIAL S/A
View of the PHB Pilot Plant for 50 tons per year
Producing Micro-organisms: Wautersia eutropha


PHYSICAL PROPERTIES
OF PHB PRODUCED BY PHBISA
PROPERTIES
Molecular weight (Da) 250.000 – 800.000*
Specific Density at 25 °C (g/cm³) 1,2
Melting point (°C) 169 - 172
Glass Transition Temperature (°C) 1 - 5
Decomposition Temperature (°C) 250
Crystallinity (%) 70
Specific Heat (J/kg.°C) 1,42
* depending on fermentation regime chosen

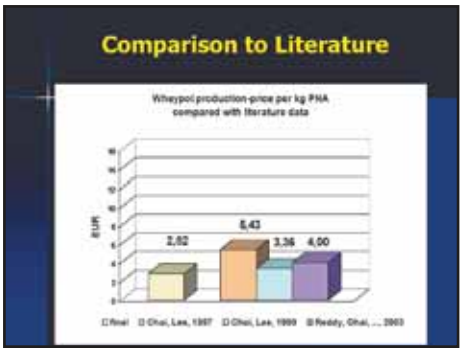

THERMOMECHANICAL PROPERTIES
PHB Homopolymer, MW (GPC) 450.000 Da
PHB plus „Thermal Stability Packaging"
Properties
Tensile Modulus (GPa, measure of the stiffness) 2,4
Tensile strength at break (MPa) 33,0
Elongation at break (%) 9,0
Notched Izod Impact (J/m, impact resistance) 26,3
Crystallinity (%) 51,0
High Distortion Temperature (°C) 75,0 – 80,0
Melt Flow Index (g/10 min,measure of the ease of flow of the polymer melt) 5,5 – 11,0

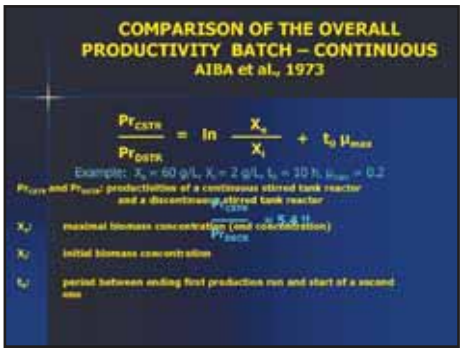

WHEYPOL: PHA Production from Surplus Whey
Dairy industry waste is a potential source of biologically-produced polymers with commercial applications in packaging. WHEYPOL is seeking a cost-effective method to tap this abundant and sustainable resource.
http://news.cec.eu.int/comm/research/industrial_technologies/articles/article_805_en.html
Whey production in Europe: 40,420.800 tons/y
Surplus WHEY: 13,462.000 tons/y
Lactose: 619.250 tons /y
205.000 t PHA/y

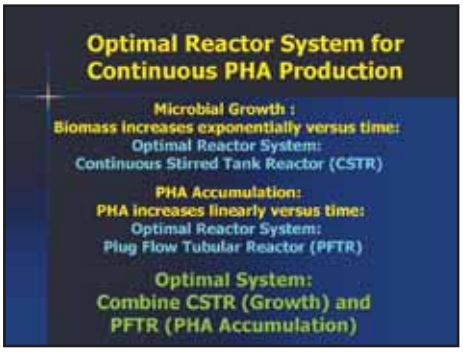

Producing Strain:
Haloferax mediterraneii DSM 1411
Osmophilic Archaebacterium demanding high salinities (ca. 150 g/L NaCl)
Copolyester production from hexoses without a precursor!!!
High Molecular weight to be expected! (Depolymerase not active!)
Triggering of co-polyester composition by precursors („tailoring" of polyester)

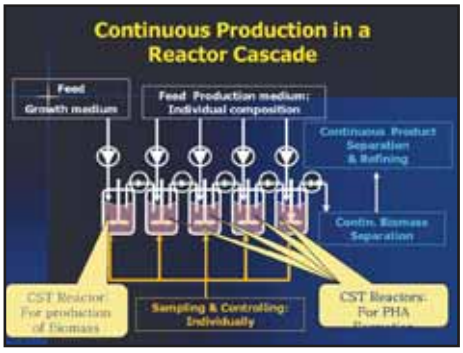

Direct Conversion of Glucose and Galactose from Hydrolyzed Whey
A High Quality Terpolyester is produced:
Mol. weight: ca. 1,5 x 10⁶
Mw/Mn= Polydispersity index: 1,1 – 1,2
By feeding propionic acid as a precursor Poly-3-HB-co-3-HV is synthesized


History of detailed operating costs
24th month
30th month
+ Improvement of yield
+ Reduced culture time
EUR
per kg produced PHA

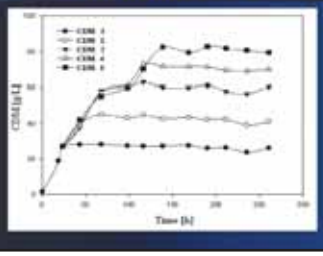

Comparison to Literature
Wheypol production-price per kg PHA compared with literature data
EUR
2,82
8,43
3,36
4,00


COMPARISON OF THE OVERALL PRODUCTIVITY BATCH – CONTINUOUS
AIBA et al., 1973
maximal biomass concentration (end concentration)
initial biomass concentration
period between ending first production run and start of a second one


Optimal Reactor System for Continuous PHA Production
Microbial Growth :
Biomass increases exponentially versus time:
Optimal Reactor System:
Continuous Stirred Tank Reactor (CSTR)
PHA Accumulation:
PHA increases linearly versus time:
Optimal Reactor System:
Plug Flow Tubular Reactor (PFTR)
Optimal System:
Combine CSTR (Growth) and PFTR (PHA Accumulation)


Continuous Production in a Reactor Cascade
Feed
Growth medium
Feed Production medium:
Individual composition
Continuous Product Separation & Refining
Contin. Biomass Separation
CST Reactor: For production of Biomass
Sampling & Controlling: Individually
CST Reactors: For PHA

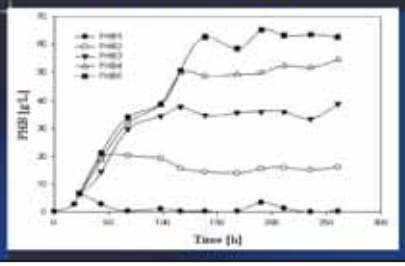
Biomass Concentration in the Fermentors 1 - 5



Feed rates (F), working volumes (V), dilution rates (D) and residence times (RT)

Reactor	F [mL/h]	V [L]	D [1/h]	RT [h]
R1	222.0	1.6	0.139	7.2
R2	19.6	1.6	0.148	6.7
R3	22.3	1.7	0.159	6.3
R4	21.9	1.7	0.167	6.0
R5	20.5	2.4	0.130	7.7
Total		9.0		33.9

Polymer Concentration in the Fermentors 1 - 5



Results

Reactor	CDM (g/L)	%PHB	PHB (g/L)	RB (g/L)	Q_x (g/Lh)	Q_P (g/Lh)	q_P (g/gh)
R1	26	4	1.0	25	3.64	0.14	0.005
R2	42	37	15	27	2.31	2.12	0.080
R3	59	60	36	24	2.79	3.27	0.139
R4	71	72	51	20	1.87	2.54	0.130
R5	81	77	63	19	1.37	1.50	0.081
Total	81	77	63	19	2.39	1.85	0.100

CDM: Cell Dry Mass %PHB: intracellular PHB content
PHB: PHB concentration RB: Residual Biomass concentration
Q_x,P: Volumetric growth rate, production rate q_P: specific production rate

Molecular mass and physical properties of the isolated PHB

Sample	M_w [kDa]	PDI	T_m [°C]	T_g [°C]	X_c [%]
F5	665 ± 1	2.6	178	2.9	68

M_w: average weight molecular mass PDI: polydispersity index
T_m: melting temperature T_g: glass transition temperature
X_c: degree of crystallinity

Results

CDW [g/L]	% PHB	PHB [g/L]	RB [g/L]	Q_x [g/L.h]	Q_P [g/L.h]	q_P [g/gh]	$Y_{x/s}$	$Y_{P/s}$
81	77	63	19	2.39	1.85	0.100	0.37	0.29

CDW, %PHB, PHB, and RB: cell dry mass, intracellular PHB content, PHB concentration, and residual biomass in the cascade outflow
Q_x,Q_P: overall volumetric productivities for biomass and PHB
q_P: specific PHB production rate
$Y_{x/s}$ and $Y_{P/s}$: biomass and PHB yield coefficients from glucose

Comparison of multistage PHB production with *C. necator*

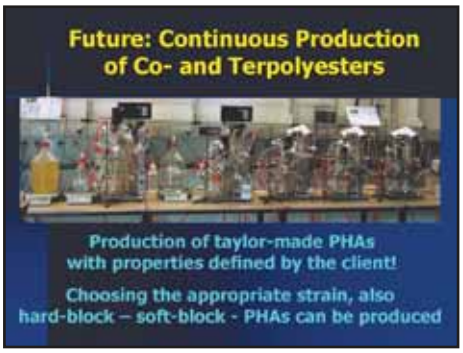
Reference	Nr. of Reactors	D1 (h^{-1})	CDW [g/L]	PHB [g/L]	% PHB	Q_P [g/Lh]
Du et al., 2001	2	0.075	42	31	72	1.23
This work	5	0.139	81	63	77	1.85

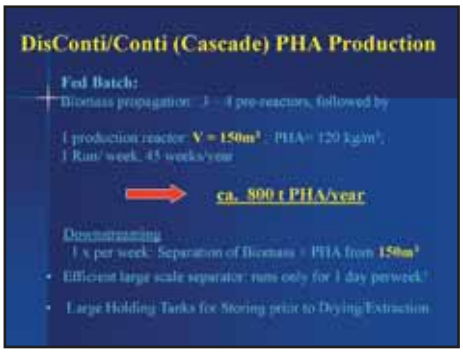
D1: dilution rate in the first stage; CDW, PHB and %PHB: cell dry weight, PHB concentration, and intracellular PHB content in the system outflow;
Q_P is overall volumetric productivity for PHB

Comparison of Industrial PHB Production with Data from the Reactor Cascade

Reference	Mode	Process Time [h]	CDM (g/L)	PHB [g/L]	% PHB	Q_P (g/Lh)	q_P (g/gh)
Nonato et al., 2001	Fed-batch	45-50	120-130	72-105	65-70	1.44	0.033
This work	5-stage continuous	34	81	63	77	1.85	0.100

CDM, PHB and %PHB: cell dry mass, PHB concentration and intracellular PHB content in the system outflow; QP: overall volumetric productivity for PHB; qP: specific PHB production rate

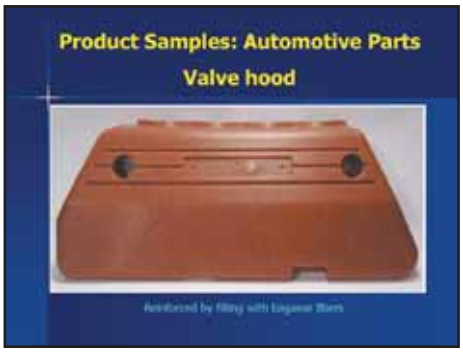

Future: Continuous Production of Co- and Terpolyesters
Production of taylor-made PHAs with properties defined by the client!
Choosing the appropriate strain, also hard-block – soft-block - PHAs can be produced


DisConti/Conti (Cascade) PHA Production
Fed Batch:
ca. 800 t PHA/year

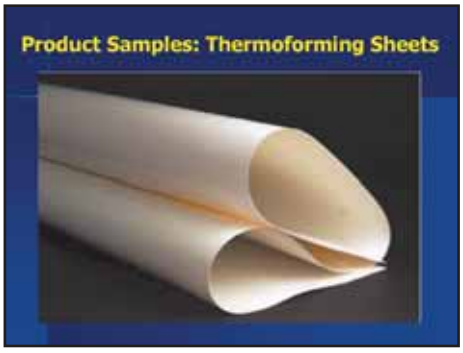

Disconti/Conti (Cascade) PHA Production
Cascade
PHA Production: 5 Reactors of about 3,6 m³ each
ca. 800 t PHA/a


Product Samples for Packaging
Powder containers
Perfume flasks


Product Samples: Pens


Product Samples: Automotive Parts
Valve hood


Product Samples: Fibres & Foams


Product Samples: Thermoforming Sheets

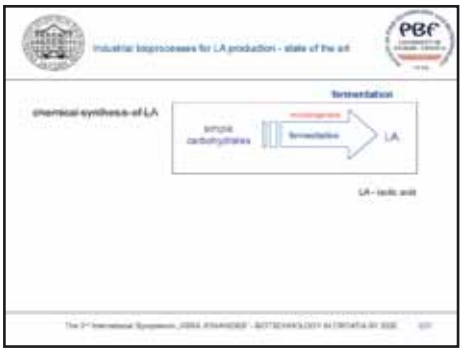

Thank You Very Much for Your Attention!

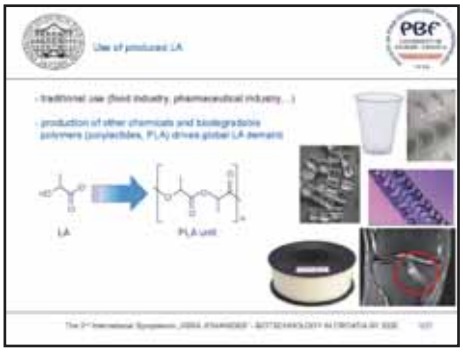
project „Use of integrated bioprocesses in production of lactic acid" (058-0581990-1997, Key Researcher Srđan Novak, Full Professor)
supported by Ministry od Science, Education and Sports of the Republic of Croatia

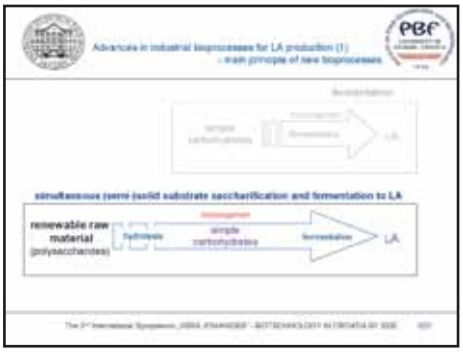
Advances in biotechnological processes for lactic acid production

Anita Slavica, Antonija Trontel, Božidar Šantek, Srđan Novak

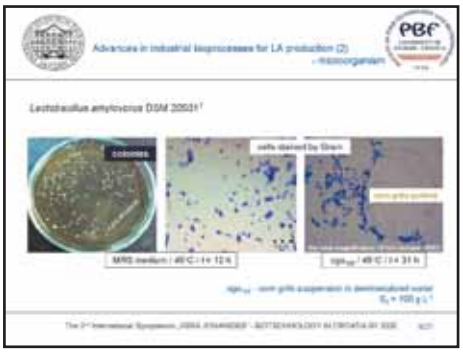
The 2nd International Symposium „VERA JOHANIDES" - BIOTECHNOLOGY IN CROATIA BY 2020





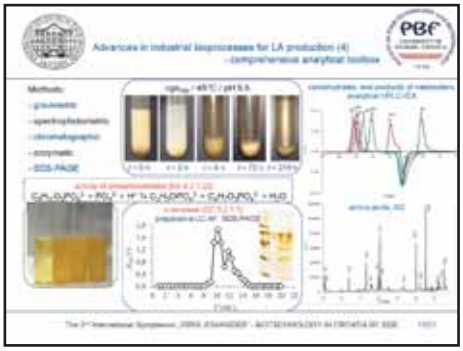

Advances in industrial bioprocesses for LA production (1)
- main principle of new bioprocesses
simultaneous (semi-)solid substrate saccharification and fermentation to LA
renewable raw material (polysaccharides)
hydrolysis
simple carbohydrates
fermentation
LA

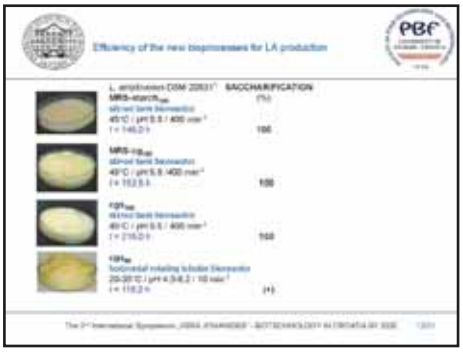

Advances in industrial bioprocesses for LA production (2)
- microorganism
Lactobacillus amylovorus DSM 20531T - lactic acid bacterium with proteolytic, amylolytic and fermentative activity

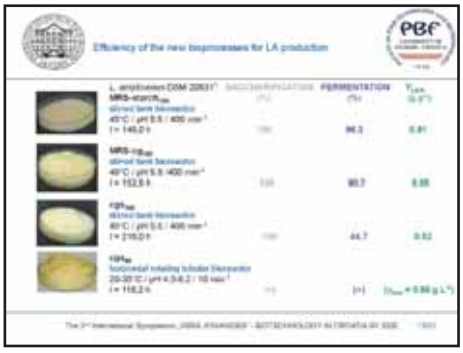

Advances in industrial bioprocesses for LA production (2)
- microorganism
Lactobacillus amylovorus DSM 20531T
colonies
cells stained by Gram
MRS medium / 40°C / t = 12 h
cgs / 49°C / t = 31 h

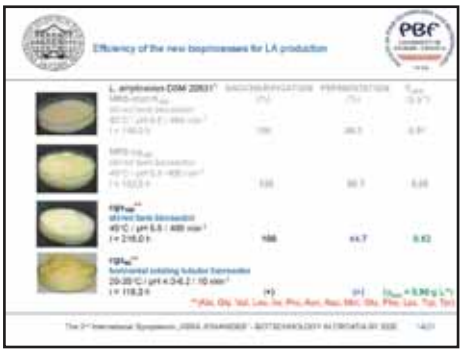

Advances in industrial bioprocesses for LA production (3)
- bioreactors and media
stirred tank bioreactor
horizontal rotating tubular bioreactor


Advances in industrial bioprocesses for LA production (3)
- bioreactors and media
media: MRS-starch
MRS-cgs
cgs
medium cgs


Advances in industrial bioprocesses for LA production (4)
- comprehensive analytical toolbox
Methods:
- gravimetric
- spectrophotometric
- chromatographic
- enzymatic
- SDS-PAGE


Efficiency of the new bioprocesses for LA production
L. amylovorus DSM 20531T
SACCHARIFICATION
(%)
MRS-starch
MRS-cgs
cgs
cgs
100
100
100
(+)


Efficiency of the new bioprocesses for LA production
L. amylovorus DSM 20531T
FERMENTATION
(%)
MRS-starch
MRS-cgs
cgs
cgs
96.3
90.7
44.7
(+)


Efficiency of the new bioprocesses for LA production


Youth on the move
Bachelor of Biotechnology
Master of Bioprocess Engineering or
Master of Molecular Biotechnology
PhD
Antonija Trontel

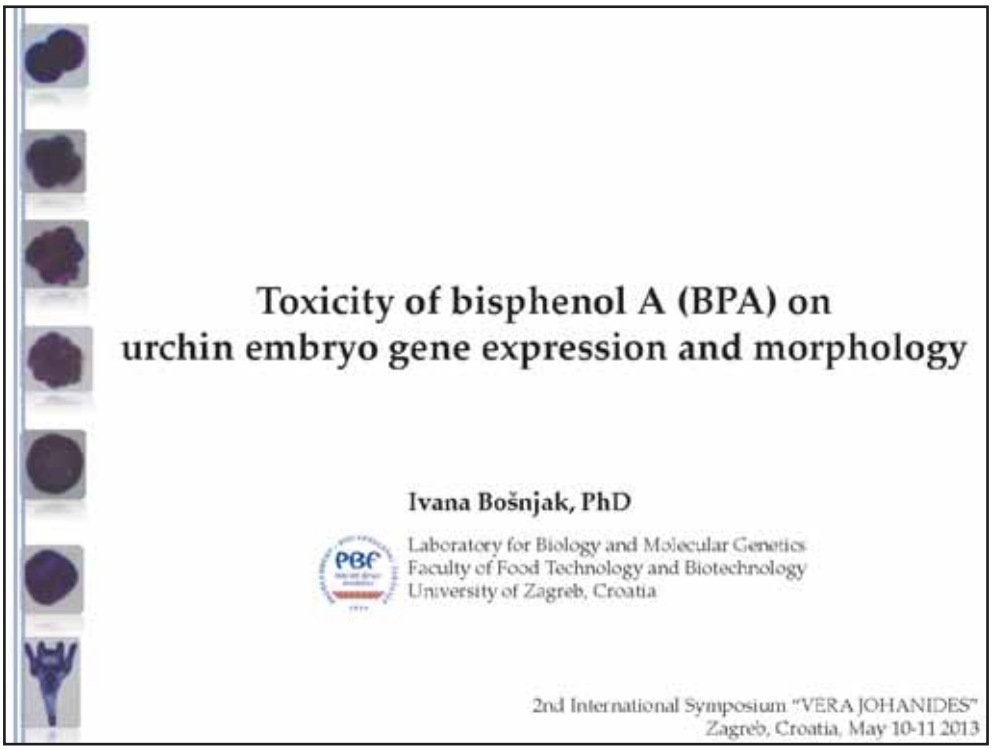

Where the new bioprocess for LA production is now (1)
Innovative bioprocess was developed √
PRIJAVA PATENTA

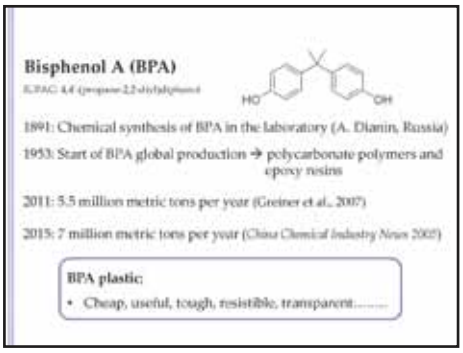

Where the new bioprocess for LA production is now (2)
Innovative bioprocess was developed √
More resource efficient, greener and competitive
bioprocess was developed √


Where the new bioprocess for LA production is now (3)
Innovative bioprocess was developed √
More resource efficient, greener and competitive
bioprocess was developed √
The performance of education system was enhanced
and the entry of young people to the labour market was
facilitated (Youth on the move) √

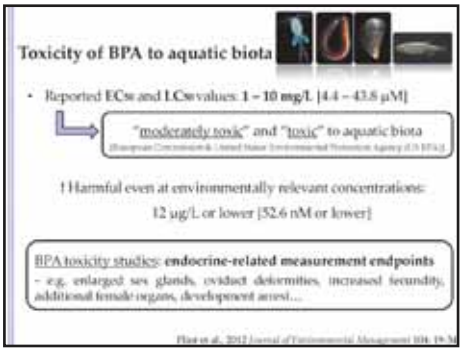

Where the new bioprocess for LA production is now (4)
The project 058-0581990-1997 is following
EUROPE2020
A European strategy for smart, sustainable and inclusive growth.

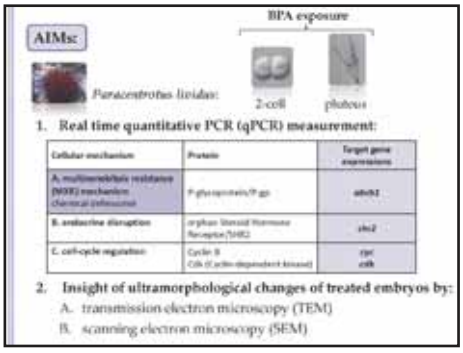

Thank you for your attention!

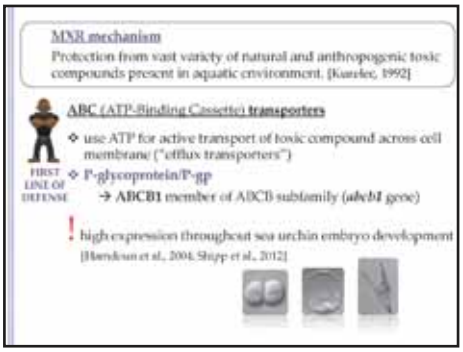

Toxicity of bisphenol A (BPA) on
urchin embryo gene expression and morphology
Ivana Bošnjak, PhD
Laboratory for Biology and Molecular Genetics
Faculty of Food Technology and Biotechnology
University of Zagreb, Croatia
2nd International Symposium "VERA JOHANIDES"
Zagreb, Croatia, May 10-11 2013

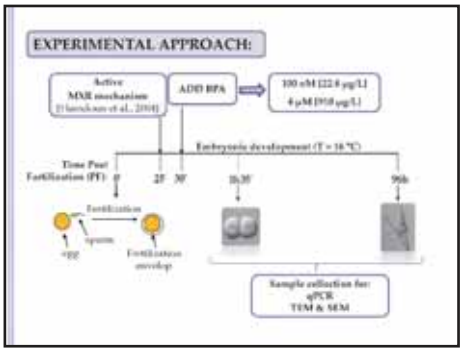

Bisphenol A (BPA)
1891: Chemical synthesis of BPA in the laboratory (A. Dianin, Russia)
1953: Start of BPA global production → polycarbonate polymers and epoxy resins
2011: 5.5 million metric tons per year (Greiner et al., 2007)
2015: 7 million metric tons per year (China Chemical Industry News 2005)
BPA plastic:
• Cheap, useful, tough, resistible, transparent.....

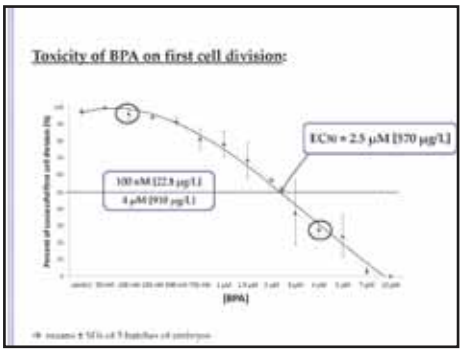

Ziploc
07

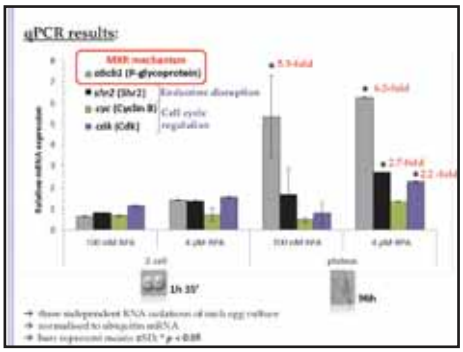

Toxicity of BPA to aquatic biota
• Reported EC50 and LC50 values: 1 – 10 mg/L [4.4 – 43.8 μM]
"moderately toxic" and "toxic" to aquatic biota
! Harmful even at environmentally relevant concentrations:
12 μg/L or lower [52.6 nM or lower]
BPA toxicity studies: endocrine-related measurement endpoints
– e.g. enlarged sex glands, oviduct deformities, increased fecundity, additional female organs, development arrest...

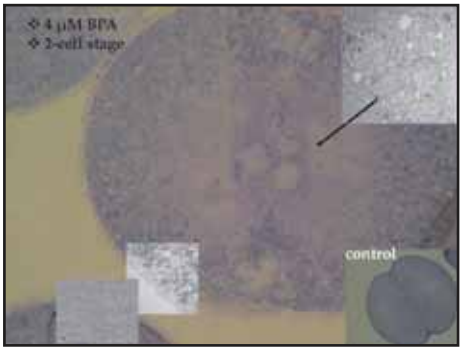

AIMs:
BPA exposure
Paracentrotus lividus:
2-cell
pluteus
1. Real time quantitative PCR (qPCR) measurement:
2. Insight of ultramorphological changes of treated embryos by:
A. transmission electron microscopy (TEM)
B. scanning electron microscopy (SEM)

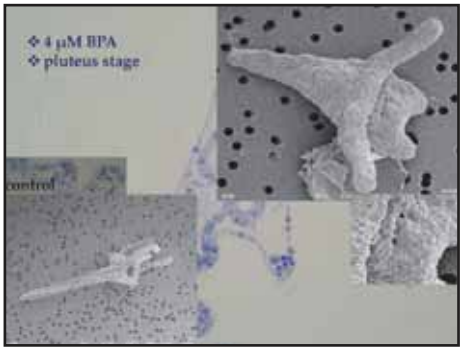

MXR mechanism
Protection from vast variety of natural and anthropogenic toxic compounds present in aquatic environment. [Kurelec, 1992]
ABC (ATP-Binding Cassette) transporters
use ATP for active transport of toxic compound across cell membrane ("efflux transporters")
FIRST LINE OF DEFENSE
P-glycoprotein/P-gp
→ ABCB1 member of ABCB subfamily (abcb1 gene)
high expression throughout sea urchin embryo development
[Hamdoun et al., 2004; Shipp et al., 2012]

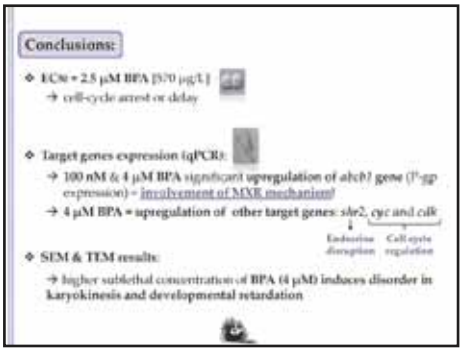

EXPERIMENTAL APPROACH:
Active MXR mechanism
ADD BPA
100 nM [22.8 µg/L]
4 µM [910 µg/L]
Embryonic development (T = 16 °C)
Time Post Fertilization (PF):
Fertilization
Fertilization envelope
Sample collection for: qPCR TEM & SEM


Toxicity of BPA on first cell division:
EC50 = 2.5 µM [570 µg/L]
100 nM [22.8 µg/L]
4 µM [910 µg/L]
[BPA]

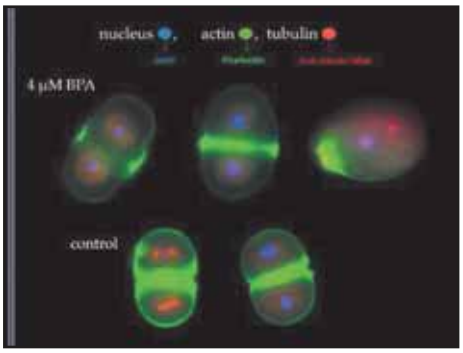

qPCR results:
MXR mechanism
abcb1 (P-glycoprotein)
efflux
Endocrine disruption
Cell cycle regulation
5.3-fold
6.2-fold
2.7-fold
2.2-fold
2 cell
pluteus
1h 35'
96h


4 µM BPA
2-cell stage
control


4 µM BPA
pluteus stage
control


Conclusions:
EC50 = 2.5 µM BPA [570 µg/L]
→ cell-cycle arrest or delay
Target genes expression (qPCR):
→ 100 nM & 4 µM BPA significant upregulation of abcb1 gene (P-gp expression) = involvement of MXR mechanism!
→ 4 µM BPA = upregulation of other target genes: slr2, cyc and cdk
Endocrine disruption
Cell cycle regulation
SEM & TEM results:
→ higher sublethal concentration of BPA (4 µM) induces disorder in karyokinesis and developmental retardation


Acknowledgements:
ASSEMBLE
Ivona Mladineo, PhD
Maria Ina Arnone, PhD
Rossella Annunziata , PhD
Marco Borra, PhD
Giovanna Benvenuto, PhD
Davide Ciaramello, facility technician


nucleus, actin, tubulin
4 μM BPA
control


BPA is toxic!
Endocrine disruptor:
• Cause hormone chaos
• Metabolism disorder, immunity disorder, affects growth and development during childhood, affects behavior, nerve and cardiovascular system disorder, cause breast cancer and prostate cancer, thyroid gland disorder .....
2008: Canada
2009: USA
2011: Europe
Embargo for BPA
in baby bottles!

The 2nd International Symposium "Vera Johanides"
BIOTECHNOLOGY IN CROATIA BY 2020

Removal of heavy metals and phosphates from wastewater by bioparticles

Lucija Nuskern, mag. oecol.
Jasna Hrenović, PhD

Zagreb, 10 – 11 May, 2013

1. Introduction

- Need for wastewater treatment prior to release into receiving environment
  - Heavy metals – toxic activity, bioacumulation
  - Phosphates – eutrophication
- BIOPARTICLE – particle composed of inorganic carrier and biofilm of bacteria
  - higher density of bacterial population, bacteria protected from biotic and abiotic stresses, no bacterial wash-off
  - better treatment efficiency

Aim of the study

- Design bioparticles able to:
  1) tolerate exposure to high concentrations of Cu^{2+}, Zn^{2+} and Ni^{2+}
  2) Simultaneously remove those heavy metals and phosphates (PO_4^{3-}) from wastewater

2. Materials and methods

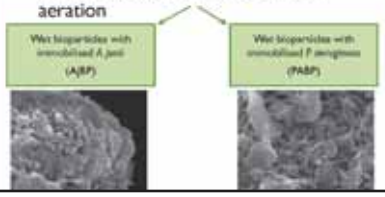
- Natural zeolitic tuff (Vranjska Banja, Srbija) – carrier material

- Bacteria:
  Acinetobacter junii DSM 1531
  - P - accumulation

  Pseudomonas aeruginosa DSM 15980
  - Heavy metal tolerance

Bacterial immobilisation

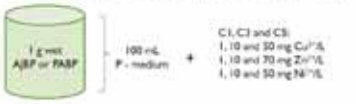
- Incubation of zeolitic tuff with pure bacterial culture of *A. junii* or *P. aeruginosa* in liquid medium, 24 h/30 °C with aeration



Experimental procedure

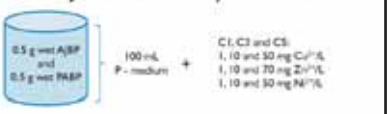
1. Planctonic bacteria

2. Immobilised bacteria (bioparticles - BP)

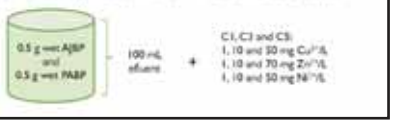


Experimental procedure

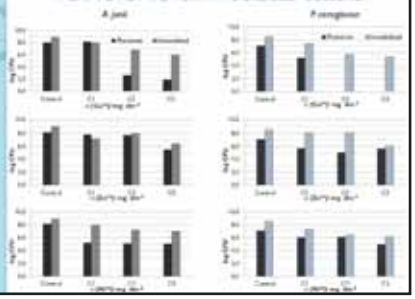
3. Mixed AJBP and PABP in synthetic wastewater



4. Mixed AJBP and PABP in real wastewater

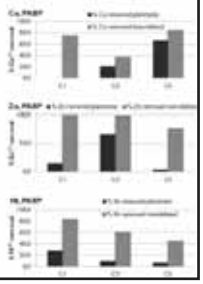


3. RESULTS - Heavy metal tolerance
PLANCTONIC vs. IMMOBILISED bacteria



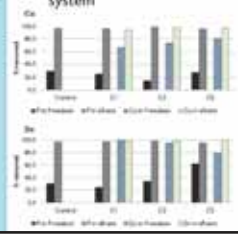
3. RESULTS - Heavy metal removal
PLANCTONIC vs. IMMOBILISED bacteria

- Highest heavy metal removal was achieved with PABP: 84,1 % Cu^{2+}, 100 % Zn^{2+} and 83,7 % Ni^{2+}



3. RESULTS
Mixed AJBP and PABP in real wastewater

- AJBP and PABP survived, multiplied and removed heavy metals and phosphates when in the same system



- Mixed AJBP and PABP removed heavy metals and phosphates from real wastewater more efficiently than from synthetic wastewater

4. CONCLUSION

- Bioparticles composed of immobilised bacteria *A. junii* and *P. aeruginosa* on natural zeolitic tuff could be used to simultaneously remove heavy metals and phosphates from wastewater

THANK YOU FOR YOUR ATTENTION!

Centre of Exellence

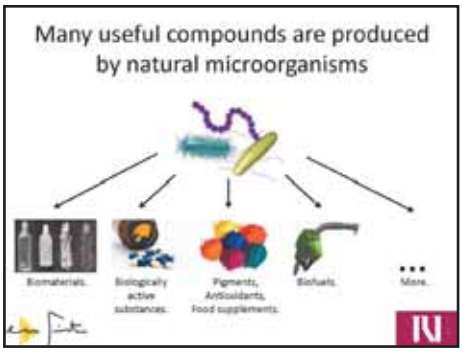
DNA guided assembly line

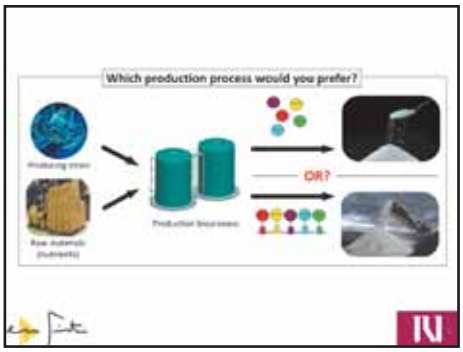
Rok Gaber

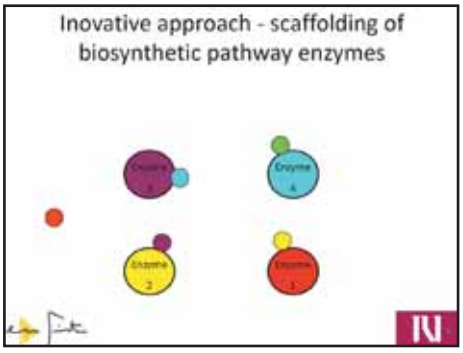
National Institute of Chemistry Slovenia

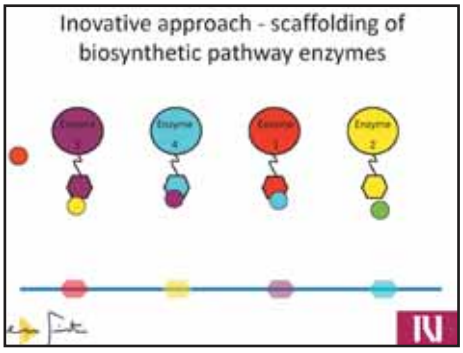
Laboratory of Biotechnology

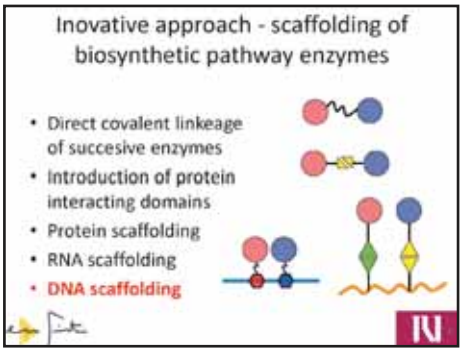
The 2nd International Symposium Vera Johanides
Biotechnology in Croatia by 2020
11.5.2013

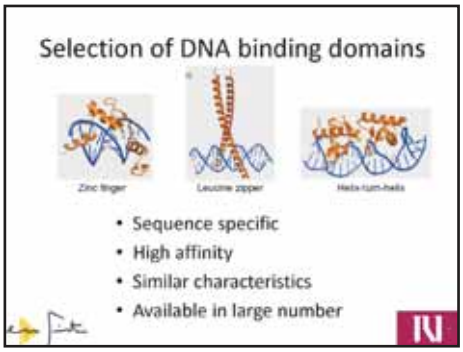


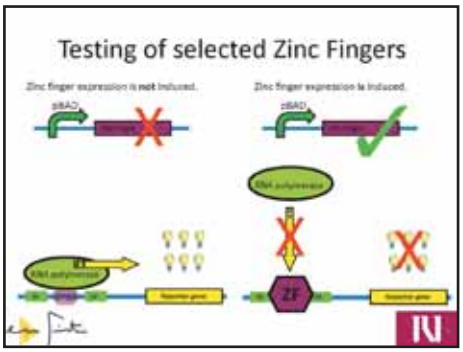


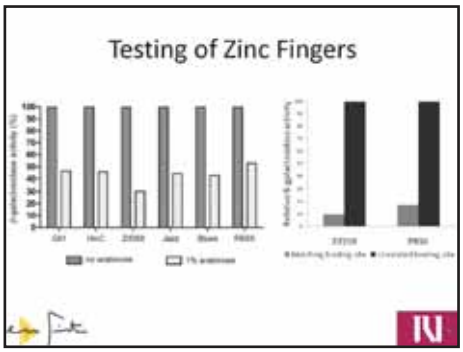




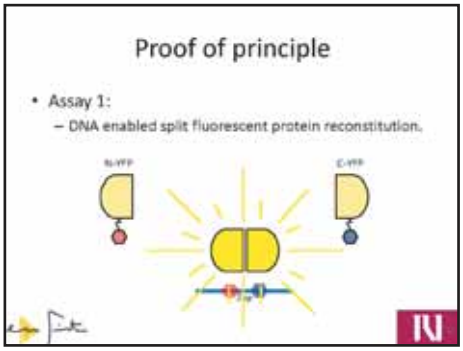

Inovative approach - scaffolding of biosynthetic pathway enzymes
• Direct covalent linkeage of succesive enzymes
• Introduction of protein interacting domains
• Protein scaffolding
• RNA scaffolding
• DNA scaffolding

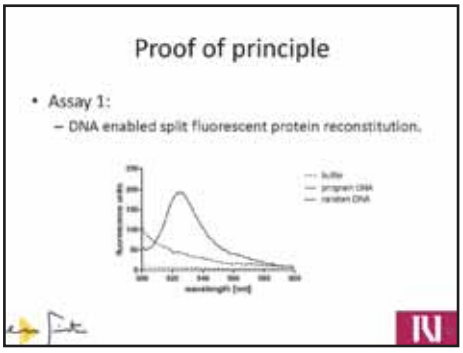

Selection of DNA binding domains
Zinc finger
Leucine zipper
Helix-turn-helix
• Sequence specific
• High affinity
• Similar characteristics
• Available in large number

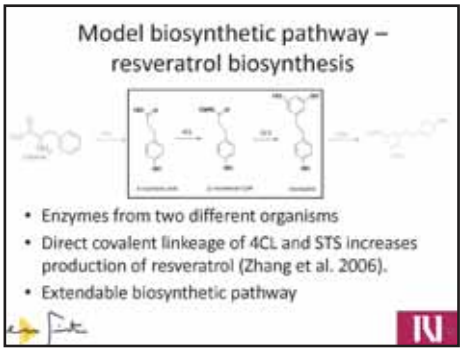

Testing of selected Zinc Fingers
ZF

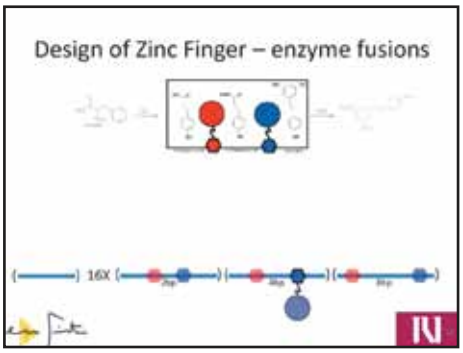

Testing of Zinc Fingers

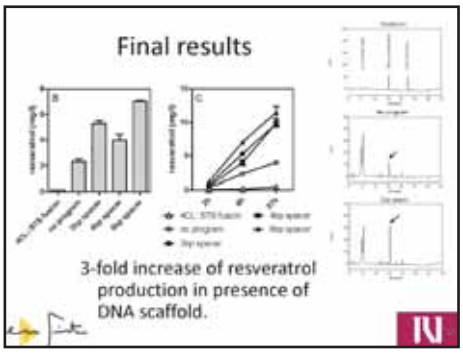

Proof of principle
• Can Zinc Fingers simultaneously bind to adjacent binding sites?


Proof of principle
• Assay 1:
– DNA enabled split fluorescent protein reconstitution.


Proof of principle
• Assay 1:
– DNA enabled split fluorescent protein reconstitution.


Model biosynthetic pathway – resveratrol biosynthesis
• Enzymes from two different organisms
• Direct covalent linkeage of 4CL and STS increases production of resveratrol (Zhang et al. 2006).
• Extendable biosynthetic pathway


Design of Zinc Finger – enzyme fusions
16X

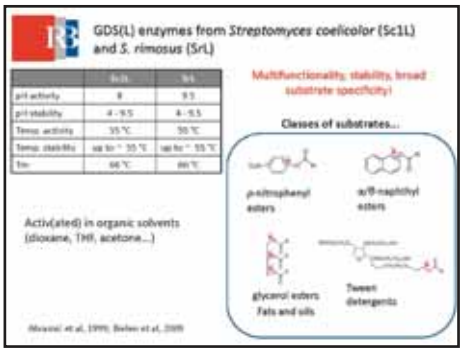

Final results
3-fold increase of resveratrol production in presence of DNA scaffold.

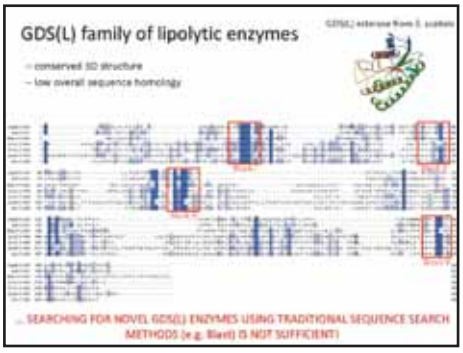

Acknowledgements and contributions

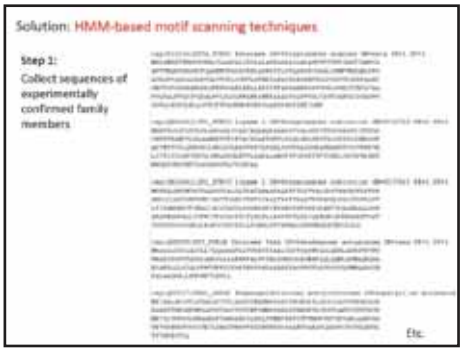
Scanning for genes encoding GDS(L) hydrolases in Actinobacteria from wide diversity of ecological niches

Bielen Ana

The 2nd International Symposium "VERA JOHANIDES"
Zagreb, May 10-11, 2013

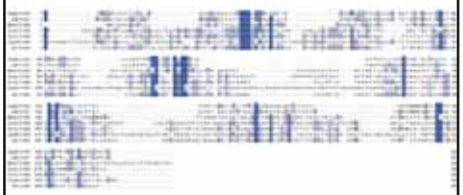






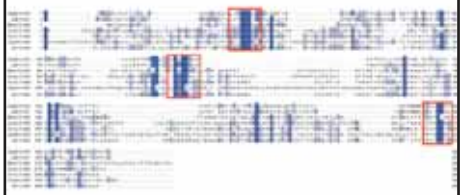
Solution: HMM-based motif scanning techniques

Step 2: Construct multiple sequence alignment (e.g. seed alignment)



Solution: HMM-based motif scanning techniques

Step 3: Cut out conserved motifs



Solution: HMM-based motif scanning techniques

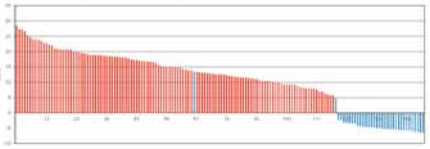
Step 4: Build model representing selected motifs (emission probabilities!)

Step 5: Scan proteomes of selected organisms for desired motifs -> select best possible motifs in every sequence in proteome and assign a score

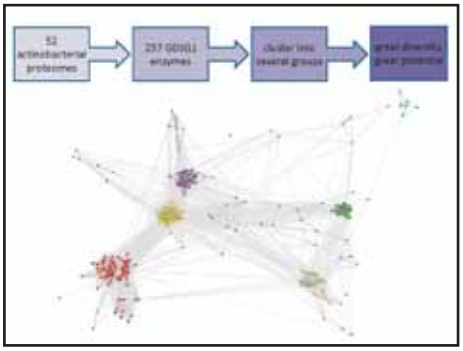
Solution: HMM-based motif scanning techniques

Step 6: Combine different motif scanning methods for optimal results -> sharp cut-off discriminates between positive and negative hits

Scores of *A. thaliana* proteome scanning for GDS(L) motifs
- Viterbi and posterior decoding combined



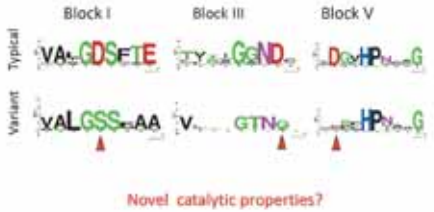
GDS(L) sequences – positive hits
Other sequences – negative hits



Evidence for expansion of genes encoding for GDS(L) hydrolases in Actinobacteria by horizontal gene transfer

1. genes on plasmids
2. deviations in codon usage
3. incongruency between GDS(L) gene-phylogeny and species phylogeny
4. close homologues in distant organisms

Previously unknown variations in motifs



Novel catalytic properties?

Conclusions

- Application of proper bioinformatic tools to explore sequence diversity present in the databases (more than blast is out there!)
- Server for motif scanning -> under construction
- Use natural diversity of hydrolitic enzymes for biotechnology

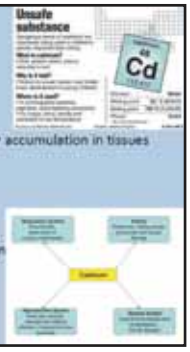
Phytoextraction of cadmium using recombinant DNA technology in maize

Mario Franić[1], Hrvoje Fulgosi[2], Lea Vojta[2], Domagoj Šimić[1]

[1] Department for breeding and genetics of maize, Agricultural institute Osijek, Osijek, Croatia.
[2] Department of molecular biology, Ruđer Bošković Institute, Zagreb, Croatia

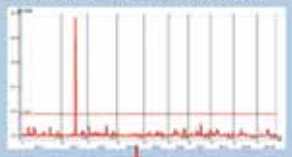
Cadmium

- Heavy metal
- Toxic at low concentrations
- Water soluble, high bioavailability → accumulation in tissues
- Health concern
- Accumulation of Cd in soil
- Expensive remediation techniques
- Adverse reactions on soil fertility
- Phytoremediation → phytoextraction

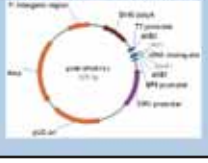


Candidate gene for cadmium accumulation in maize leaf

- **WinQTL Cartographer:** QTL - chrom2 for IBM:



- Maize genome database (www.maizegdb.org)
- Aspartate kinase (ask2)
- *Arizona Genomics Institute* → ZM_BFc003612C cDNA library
- pCMV Sport 6.1 – sequencing with 35S and T7 primers



Epitope tagging

- HA and FLAG tag addition using PCR reaction

HA tag:

HA tag Gene

5'-AGC GTA ATC TGG AAC ATC GTA TGG GTA ATG GCT GTG GAT TGT GCC ATT-3'

FLAG tag:

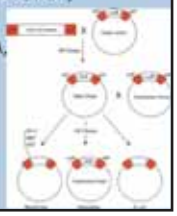
FLAG tag Linker

5'-CTA TTT GTC ATC GTC GTC CTT GTA GTC TCT GAA CTG AGC GTA ATC TGG AAC ATC GTA-3'

HA tag

Cloning strategy

- Gateway cloning (Invitrogen, USA)
- Donor vector - pENTR™/SD/D-TOPO®, (Invitrogen, USA)
- Destination vector- pANIC 6A, University of Tennessee

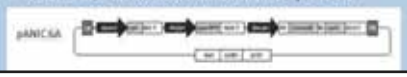


TOPO cloning

- Purification of PCR products - GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences, UK)
- Cloning the purified DNA construct (*ask2* gene with HA and FLAG tag) into a TOPO entry vector (pENTR™/SD/D-TOPO®, Invitrogen, USA)
- Transformation of *One Shot® TOP10 Chemically Competent E. coli cells* (Invitrogen, USA)

- Selection of transformants on kanamycin (50μg/mL) plates → minipreparation
- PCR, electrophoresis

- Vector suited for monocot transformation – pANIC 6A



LR reaction

- LR reaction was done according to manufacturers protocol (Invitrogen), DH5α *E.coli* cells were transformed and plated on kanamycin plates (50μg/mL)
- TOPO entry vector
- pANIC 6A destination vector

- Minipreparation of overnight cultures
- Restriction using EcoRV – cleaves the pANIC 6A vector once (16937) and *ask2* sequence once (1517)

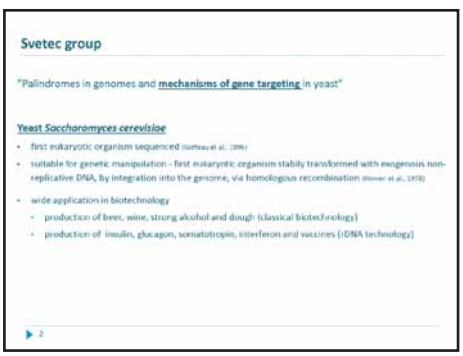
- Sequencing
- **Future steps:** expression clone→ *Agrobacterium* → maize

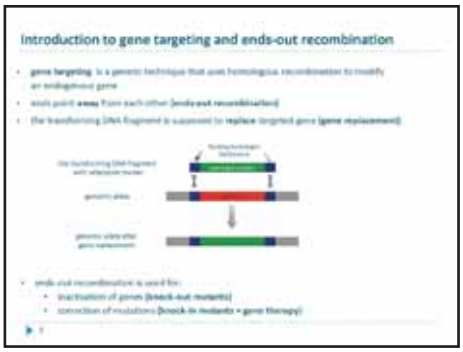
THANK YOU☺

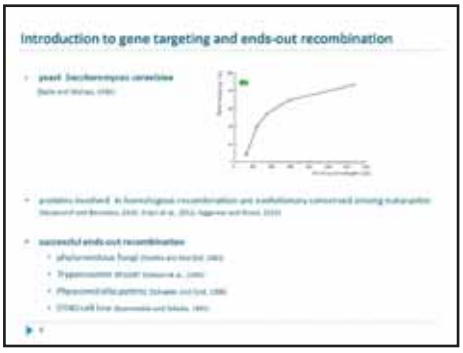
Genetic side-effects during gene replacement in yeast *Saccharomyces cerevisiae*

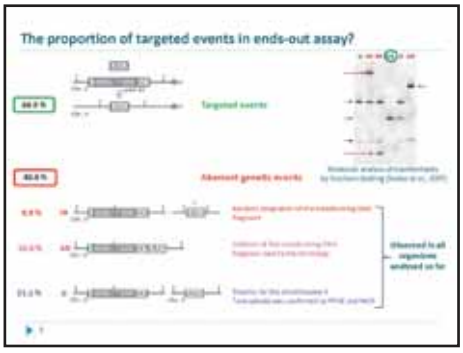
Anamarija Štafa Ph.D.

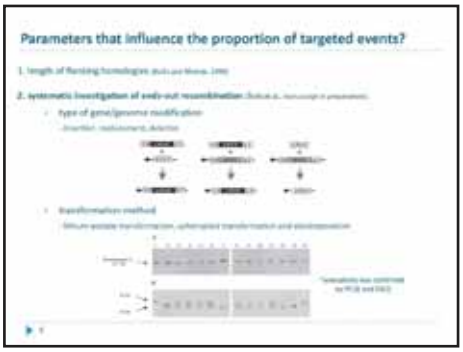
Laboratory for Biology and Microbial Genetics
Department of Biochemical Engineering
Faculty of Food Technology and Biotechnology
University of Zagreb

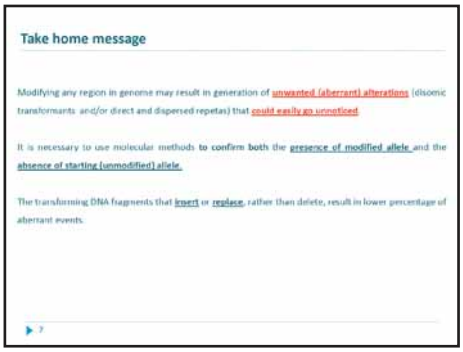






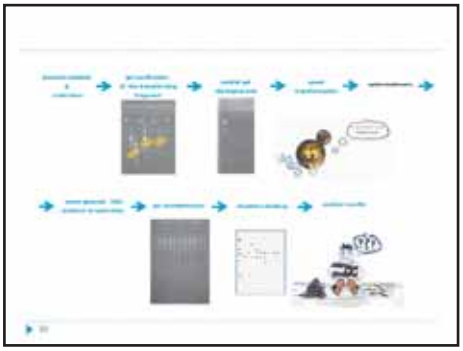



Parameters that influence the proportion of targeted events?


Take home message
Modifying any region in genome may result in generation of unwanted (aberrant) alterations (disomic transformants and/or direct and dispersed repetas) that could easily go unnoticed.
It is necessary to use molecular methods to confirm both the presence of modified allele and the absence of starting (unmodified) allele.
The transforming DNA fragments that insert or replace, rather than delete, result in lower percentage of aberrant events.


Acknowledgements:
FUNDING


Thank you for your attention



The 2nd International Symposium
"VERA JOHANIDES"

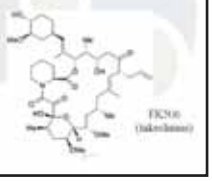
Regulation of biosynthesis of immunosuppressant FK506 (tacrolimus) by *Streptomyces tsukubaensis*

dr. Dušan GORANOVIČ

Zagreb, May 2013

FK506 (takrolimus – Tsukuba macrolide immunosuppressant)

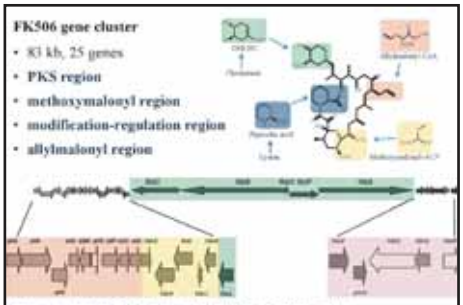
- medically important polyketide immunosuppressant, produced by *Streptomyces tsukubaensis*
- FK506 is synthesized by a hybrid PKS/NRPS system



Streptomyces tsukubaensis

FK506 gene cluster

- 83 kb, 25 genes
- **PKS region**
- **methoxymalonyl region**
- **modification-regulation region**
- **allylmalonyl region**



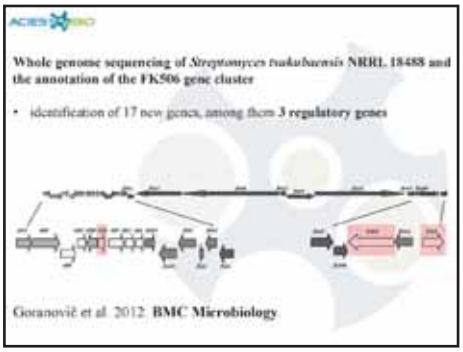
Goranović et al. 2010. **Journal of Biological Chemistry**
Kosec et al. 2012. **Metabolic Engineering**

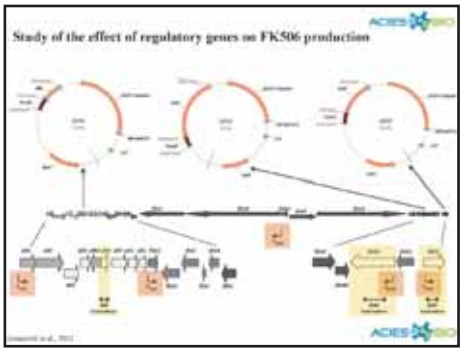
Regulation of secondary metabolite biosynthesis

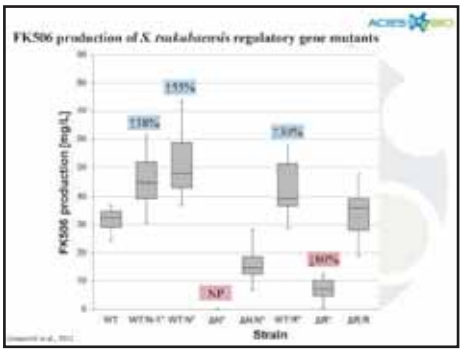
- complex regulation of secondary metabolism
- global (pleiotropic) regulation
- pathway specific regulation

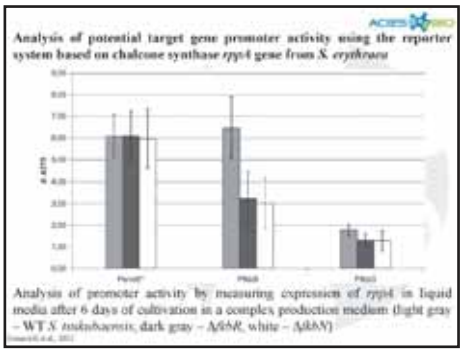
Whole genome sequencing of *Streptomyces tsukubaensis* NRRL 18488 and the annotation of the FK506 gene cluster

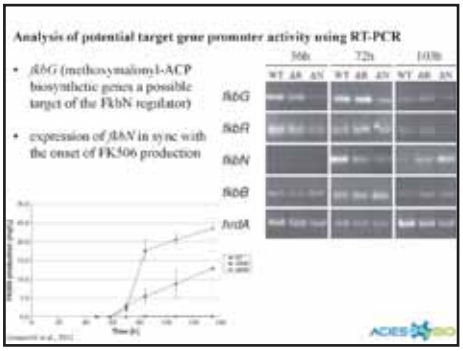
- identification of 17 new genes, among them **3 regulatory genes**


Whole genome sequencing of *Streptomyces tsukubaensis* NRRL 18488 and the annotation of the FK506 gene cluster
• identification of 17 new genes, among them **3 regulatory genes**
Goranovič et al. 2012. **BMC Microbiology**


Study of the effect of regulatory genes on FK506 production


FK506 production of *S. tsukubaensis* regulatory gene mutants
FK506 production [mg/L]
155%
138%
139%
NP
180%
Strain


Analysis of potential target gene promoter activity using the reporter system based on chalcone synthase *rppA* gene from *S. erythraea*
Analysis of promoter activity by measuring expression of *rppA* in liquid media after 6 days of cultivation in a complex production medium (light gray – WT *S. tsukubaensis*, dark gray – Δ*fkbR*, white – Δ*fkbN*)

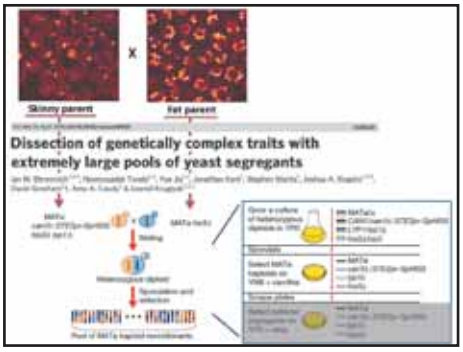

Analysis of potential target gene promoter activity using RT-PCR
• *fkbG* (methoxymalonyl-ACP biosynthetic genes a possible target of the FkbN regulator)
• expression of *fkbN* in sync with the onset of FK506 production
36h
72h
110h
WT ΔR ΔN
fkbG
fkbR
fkbN
fkbB
fkbA

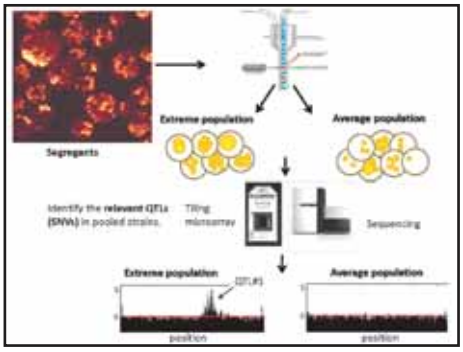

References

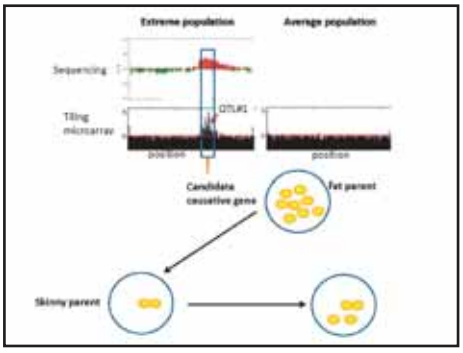
IDENTIFICATION OF GENETIC VARIANTS RESPONSIBLE FOR BIOTECHNOLOGICALLY IMPORTANT TRAITS AND THE DESIGN OF NEW GENERATION INDUSTRIAL YEAST STRAINS

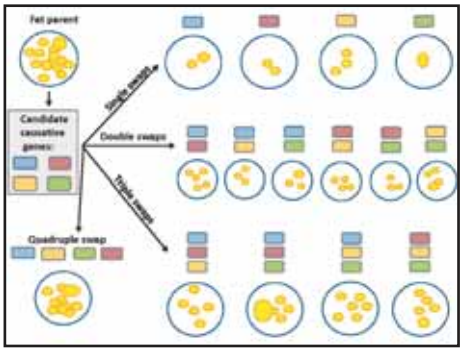
Mojca Brložnik[1], Tomaž Curk[2], Klaus Natter[3], Uroš Petrovič[1]

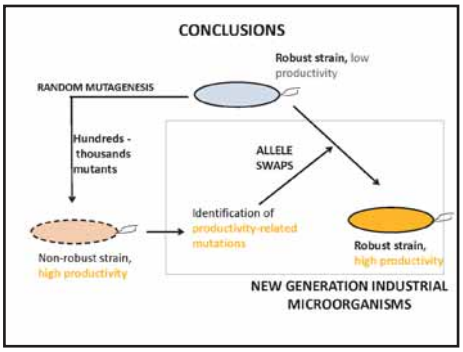
[1] Jožef Stefan Institute, Ljubljana, Slovenia.
[2] University of Ljubljana, Slovenia.
[3] University of Graz, Austria.











ACKNOWLEDGEMENTS

Tomaž Curk

Bioinformatics Laboratory
Faculty of Computer and Information Science
University of Ljubljana
Slovenia

Klaus Natter

Yeast Genetics and Molecular Biology Group
Institute of Molecular Biosciences
University of Graz
Austria

University of Ljubljana, Biotechnical Faculty
Dept. of Food Science and Technology, Chair of Microbiology

Mining bacterial genomes for laccases

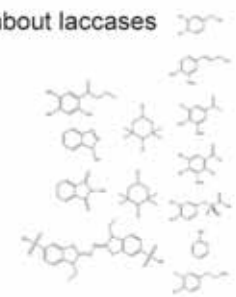
Luka Ausec*, Marko Verce, Miha Črnigoj, Vesna Jerman, **Ines Mandić-Mulec****

The 2nd International Symposium "VERA JOHANIDES", Zagreb, May 11 2013

*

Why care about laccases

- What do they do?
- How do they do it?



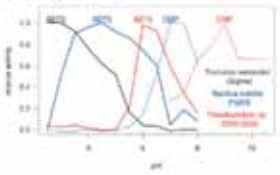
Why care about laccases

- What do they do?
- How do they do it?

- Environmentally friendly

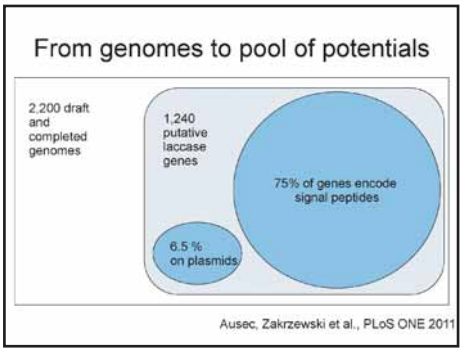
Fungal vs. Bacterial Laccases

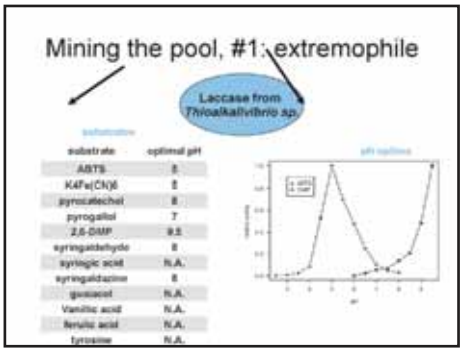
- Ease of pruduction
- Substrate range
- pH and temperature optimum
- Salt tolerance

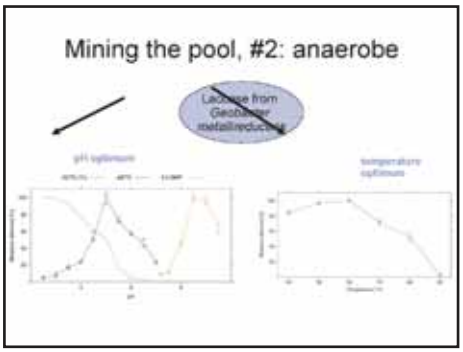


Sources of novel bacterial laccases

	DNA potential	Activty
related cultured strains	unknown	known
metagenomics	unknown	unknown
bioinformatics	known	unknown







Conclusions

- Bacterial laccases are diverse
- Bioinformatic (HMM-based) approaches successful
- Bacterial laccases have promissing traits for biotechnological applications

Thank you.

luka.ausec@bf.uni-lj.si

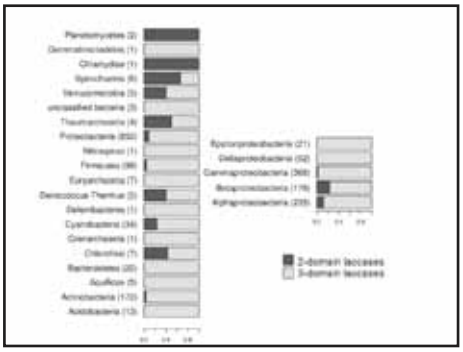
**

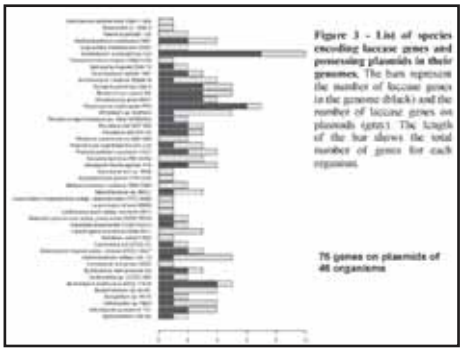
Bioinformatic analysis of bacterial laccase-like genes

>2200 completed and draft bacterial genomes

METHODS:

- profile Hidden Markov Models (pHMM)
- 5 models constructed





The 2nd International Symposium
"VERA JOHANIDES"
BIOTECHNOLOGY IN CROATIA BY 2020

Zagreb, May 10-11, 2013

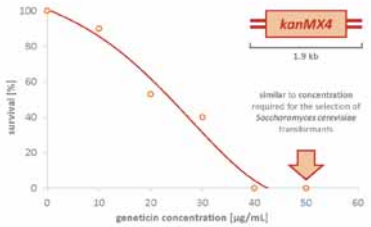
Genetic TRANSFORMATION of yeast *Dekkera/Brettanomyces bruxellensis*

Bojan Žunar
Marina Miklenić, Anamarija Štafa
Berislav Lisnić, Ivan-Krešimir Svetec

Laboratory for Biology and Microbial Genetics
Department of Biochemical Engineering
Faculty of Food Technology and Biotechnology, University of Zagreb

About *D. bruxellensis*

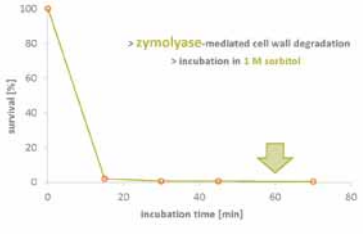
Geneticin resistance

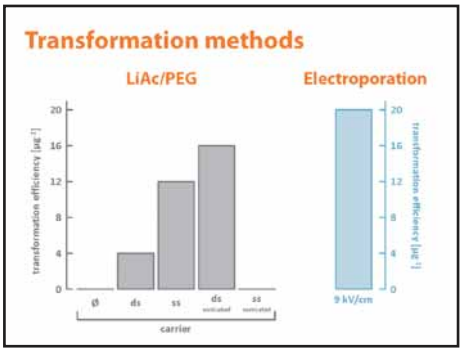


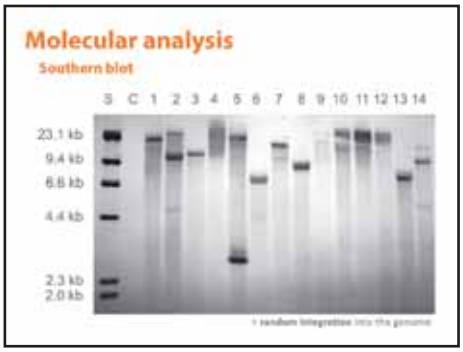
Transformation methods

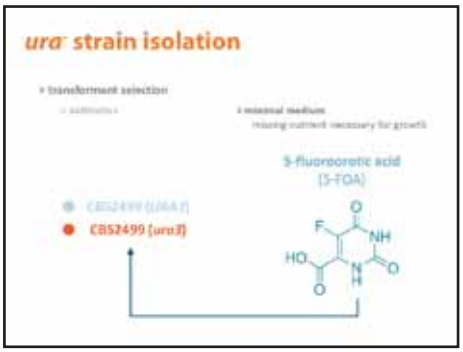
Transformation methods

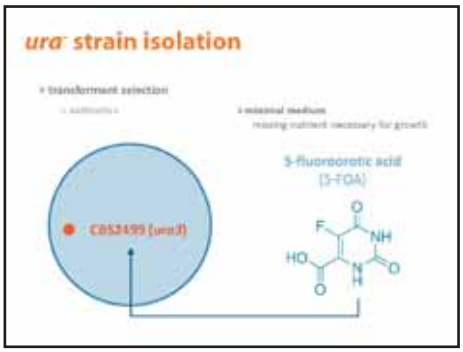
Spheroplast preparation

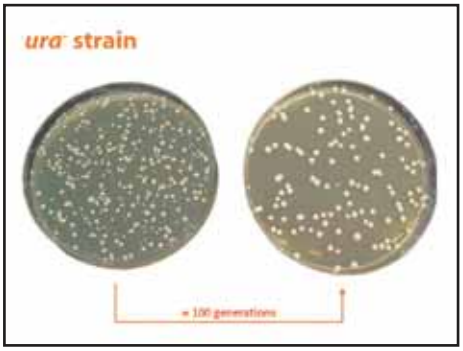


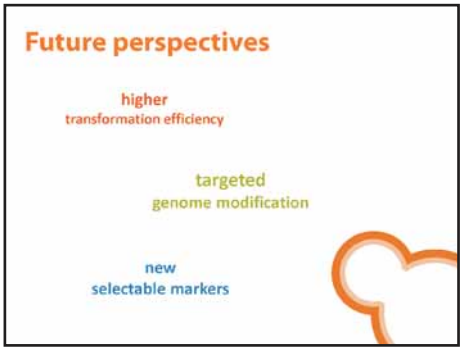

Transformation methods
LiAc/PEG
Electroporation
transformation efficiency [µg⁻¹]
transformation efficiency [µl⁻¹]
20
16
12
8
4
0
Ø
ds
ss
ds
ss
carrier
9 kV/cm

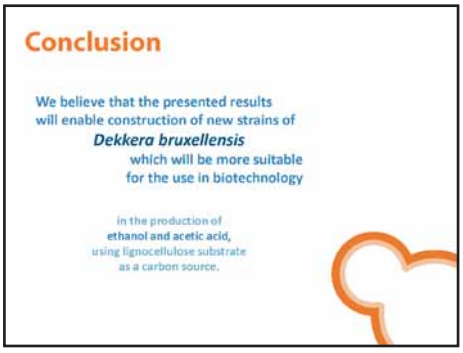

Molecular analysis
Southern blot
S C 1 2 3 4 5 6 7 8 9 10 11 12 13 14
23.1 kb
9.4 kb
6.6 kb
4.4 kb
2.3 kb
2.0 kb


ura⁻ strain isolation
5-fluoroorotic acid
(5-FOA)
CBS2499 (ura3)


ura⁻ strain isolation
5-fluoroorotic acid
(5-FOA)
CBS2499 (ura3)


ura⁻ strain
≈ 100 generations


Future perspectives
higher
transformation efficiency
targeted
genome modification
new
selectable markers


Conclusion
We believe that the presented results
will enable construction of new strains of
Dekkera bruxellensis
which will be more suitable
for the use in biotechnology
in the production of
ethanol and acetic acid,
using lignocellulose substrate
as a carbon source.

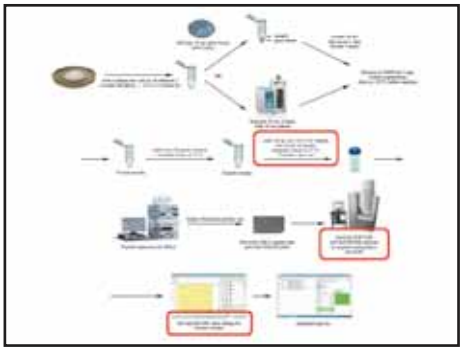

Acknowledgements
prof. Ivan-Krešimir Svetec Ph.D.
Marina Miklenić M.Sc.
Anamarija Štafa Ph.D.
Berislav Lisnić Ph.D.
Nataša Tomašević
Funding:
Ministry of Science, Education and Sport
of the Republic of Croatia
Thank you!

Mass spectrometry-based clinical proteomics

Antonio Starcevic, PhD - Faculty of Food Technology and Biotechnology (PBF), University of Zagreb
astar@pbf.hr

2/7/2014 HRZZ Installation Grants

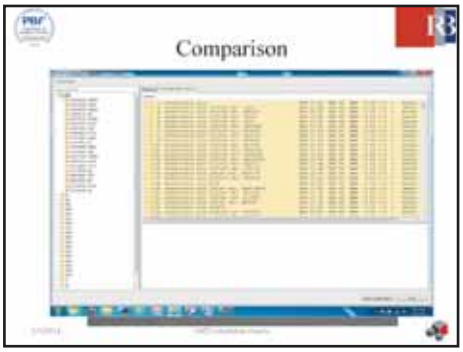
Outline



Do bacteria have fingerprints?

Features	Our CAF+/CAF- MS2 approach	Current MS1 approach (Bruker MALDI Biotyper)
Sample preparation	+	++
Consumable cost	+	++
Robustness	++	++
Reproducibility	++	+
Orthogonal	+	-
Reference library size	++	+
Resolution	+++	+
Affordability	+	++

SWOT analysis (MS vs MS/MS)


Comparison


Inventory


Results (head to head comparison)


Thanks!

ENHANCEMENT OF EFFICIENCY OF ETHANOL PRODUCTION FROM JERUSALEM ARTICHOKE

Quang D. Nguyen, Kálmán Dénes, Csilla Farkas, Ágoston Hoschke, Judit M. Rezessy-Szabó

CORVINUS UNIVERSITY OF BUDAPEST
Faculty of Food Science

The 2nd International Symposium „Vera Johanides" 2013

HUNGARY'S RENEWAL
NEW SZÉCHENYI PLAN

Production of bioethanol in EU-27 - 2009 (million liters)

Total: 3703

Welcome Croatia to be in this map (EU-28)!
On the 2nd International Symposium „Vera Johanides" in 2015
– Biotechnology and Forestry Complex in Croatia!

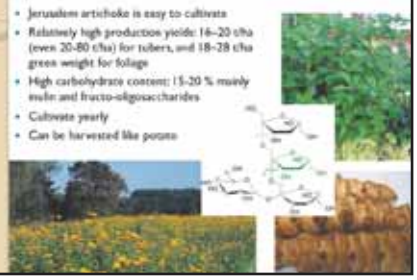
Source: www.biofuels-platform.ch

Productivity of ethanol using different raw materials

Plant	Main carbohydrate	Productivity (l/ha)
Sugarcane	saccharose	8000
Sugar beet	saccharose	3300
Jerusalem artichoke	inulin	4200
corn	starch	2100
potato	starch	1900
wheat	starch	1800
wood	cellulose/lignocellulose	?????

Potential of JA-based bioethanol production

- Jerusalem artichoke is easy to cultivate
- Relatively high production yields: 16–20 t/ha (even 20-80 t/ha) for tubers, and 18–28 t/ha green weight for foliage
- High carbohydrate content: 15-20 % mainly inulin and fructo-oligosaccharides
- Cultivate yearly
- Can be harvested like potato



Problems addressed

- *Saccharomyces cerevisiae* strain does not exhibit so high inulinase activity
- Treatment: acidic or enzymatic
- Exogenous enzyme preparation
- Low efficiency of bioconversion (60-70 %)

Objectives

Technological development for enhancement of efficiency of ethanol production using Jerusalem artichoke as raw material

- Inoculation technique
- Simultan saccharification and fermentation
- Semi-continuous fermentation technology

Development of inoculation technique

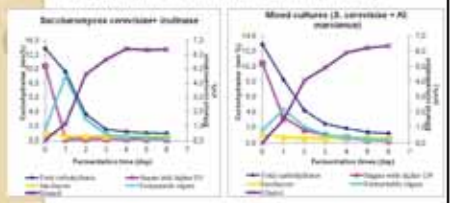
- Effects of various inoculation techniques were investigated

Best one

on initial day 2/3 amount of inoculum (cell number) and on second day: 1/3 amount

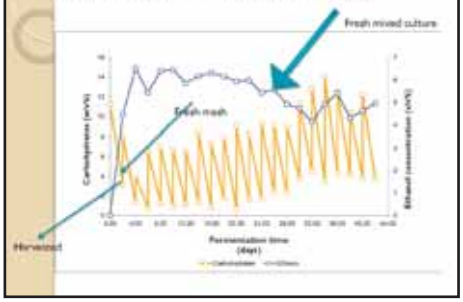
Efficiency of bioconversion: 90 %

SSF vs. Mixed Culture Fermentation



Efficiency of bioconversion: 85-88 %

Semi-continuous technology



Conclusion

The use of mixed culture in combination with semi-continuous fermentation technology definetely increases in the productivity, thus it is very promising for development of technology for production of bioethanol based on Jerusalem artichoke as raw material

Acknowledgement

This work was supported by Hungarian Development Agency through Project number TÁMOP-4.2.1./B-09/1-KMR-2010-0005 and TECH_09-A3-2009-0194, as well as Hungarian Academy of Science through Bolyai János Research Grant

Thank you for your kind attention!

Our forthcoming meeting:

The 3rd International Scientific Symposium "Vera Johanides" – Biotechnology, Forestry and Wood Technology in Croatia, Zagreb, 2015

Role of Forests in Croatia – From the Perspective of Research Institute

Dijana Vuletić*

Croatian Forest Research Institute, Jastrebarsko, Croatia

Minireview

The aim of this paper is to present importance and the role of forests for environment, society and economy. This will be presented through various international documents, policy orientations and global challenges like climate change and nature protection.
How those processes are understood in Croatia and how they can be approached by national policy, research and forest management is second part of this paper. Position of Croatian forest research institute in attempt to answer to the main challenges is presented by its research programs and main ongoing projects.
At the end Croatian forest research institute as only state public research organization in field of forestry is presented in nutshell.

Forests at global level

Forests are 'the lungs of our planet' and often regarded as symbol of the environment. They are the most diverse ecosystems on land and among the most important repositories of terrestrial biological diversity. Some rain forests are among the oldest ecosystems on Earth. Together, tropical, temperate and boreal forests offer very diverse habitats for plants, animals and micro-organisms and are therefore one of the most-important gene-reserves.

Biological diversity of forests is the basis for a wide array of goods and services to humanity which are sources of direct economic benefit and play a vital role in the daily life of rural communities in many areas, as sources of wood and non-wood products, as contributors to soil and water conservation, and as repositories of aesthetic, ethical, cultural and religious values. Timber, pulpwood, firewood, fodder, meat, cash crops, fish and medicinal plants from the forest provide livelihoods for hundreds of millions of people worldwide. But only a fraction of known species has been examined for potential medicinal, agricultural or industrial value.

Their role is crucial in Land use and land use change (LULUCF) as identified by the United Nations Climate Change Secretariat, having impact in global carbon

*Corresponding author: dijanav@sumins.com

cycle and influencing atmosphere. They are therefore a powerful tool to mitigate the climate changes, stabilize pollution and consequently global warming and protect soils and watersheds. On the top of that forests provides places for recreation and relaxation, built landscapes and create images that people and even nations holds in their memories, preserving them forever. Forests are, like in Croatia, in many basic country documents defined as resource of high national importance (Constitution of Republic of Croatia NN56/90, 135/97, 8/98 113/00, 124/00, 28/01, 41/01, 55/01, 76/10, 85/10).

Forests are threatened as any other ecosystem on Earth but still without right answer on those threats. There have been numerous efforts aimed at safeguarding the world's biodiversity by protecting species in protected areas of different levels of protection and also outside their original habitats. For example, seeds of some of the most economically important trees are being conserved in seed centers and gene-banks as a way of protecting their genetic diversity.

On global scale forests provide more than 10% of the GDP in many of the poorest countries. It is estimated that the forestry sector provides formal employment for 10 million people and informal employment for additional 30 to 50 million people in developing countries. The FAO Forestry Program focuses on how to maximize the potential of forests, trees and related resources to improve people's economic, social and environmental conditions while ensuring that the resource is conserved to meet the needs of future generations. FAO also supports the development and implementation of appropriate policies and practices.

Forests in Europe

Europe i**s the most forest-rich region in the world** representing 25% of our global forest resources. Sustainable forest management practices widely present in EU increasingly promote enhancement of biological diversity. But, diseases, extreme weather conditions like storms, and fires also threaten forests (State of Europe's Forests 2011). The decision reached by ministers to go ahead with the negotiations towards a Legally Binding Agreement on Forests represents a major step towards creating the necessary structure for a coherent approach to the continent's forests. A strengthened political cooperation in Europe will be vital to achieve a balanced and stable continuity of all environmental, economic and social forest functions and contribute to the achievement of international agreed objectives.

With goal to bring forest issue to the focus the EU Commission adopted a new Forest Strategy (2013) which responds to the new challenges facing forests and the

forest sector. The EU has a long history of contributing through its policies to implementing sustainable forest management and to Member States' decisions on forests. The most important developments include:

- the Europe 2020 strategy for growth and jobs,
- the Resource Efficiency Roadmap,
- Rural Development Policy,
- Industrial Policy,
- the EU Climate and Energy Package with its 2020 targets,
- the Plant Health and Reproductive Materials Strategy and
- the Biodiversity and Bio-economy Strategies.

Following a new approach, the Strategy "goes out of the forest", addressing aspects of the **value chain** i.e. the way forest resources are used to generate goods and services strongly influence forest management. The Strategy highlights that forests are not only important for rural development, but also for the **environment** – especially for biodiversity; forest-based industries; **bioenergy**; and **fighting against climate change**. Stressing the need of a **holistic approach**, it also emphasizes that impacts of other policies on forests as well as developments taking place beyond forest boundaries should be taken into account (EU Forest Strategy 2013).

One of most important part is that new strategy underlines that forest-linked EU policies should fully be taken into account and reflected in national forest policies.

Forests in Croatia

According to data from the new Management Plan (2006^{th}-2015^{th}) forests in the Republic of Croatia occupy 2.7 million hectares or approximately 47% of total area (Croatian forests, 2006). State owns 2.1 mil ha (78%) of forests and forest land, while 0.6 mil ha (22%) is privately owned. "Croatian forests", ltd co., Zagreb is responsible for management of majority of state forests for which they received certificate from Forest Stewardship Council (FSC), while some smaller areas are managed by other legal entities (Martinić & Dekanić, 2003).

The NFPS have been brought by Government of the Republic of Croatia in the year 2003 stressing most important economic, environmental and social functions of forests in Croatia and their major impact on the quality of life.

The overall policy aim of NFPS was (NFPS 2003):

View from Eddy covariance tower on crowns of Pedunculate oak forests near Jastrebarsko

"Through sustainable management, use and comprehensive protection of forest resources and biodiversity increase the contribution to the national economy, applying research results, while respecting international trends and local community rights." Today, although we are thinking about new NFPS which will address new challenges and changes, we can agree that this overall policy aim is still valid.

Croatian forest ecosystems as any other forests on the Earth provide multiple goods and services (especially relevant are water-related services, soil protection, and an exceptional richness in terms of biodiversity and unique non-wood forest products like aromatic plants, different fruits and berries, truffles, mushrooms, etc). These goods and services are crucial to the socio-economic development of the region's rural areas as well as to the welfare of its urban populations.

In modern context of rising concern for the environment and the climate extremes society witnessing, when the recent international scientific studies confirm that it is *extremely likely* that humanity is to blame for the global warming and climate change a clear view on the current state of scientific knowledge related to forest and forestry, continuous monitoring and implementation of the state-of the art forestry research should become national priorities.

That's way advancing scientific knowledge and fostering innovation is essential, then, to ensure the sustainable management of forests and maximise the potential role of their unique goods and services in building a knowledge-based bio-economy in the region.

Addressing the challenges from research perspective

The Croatian forest research institute has actively working on development of research programs in a way to be able to address those main global challenges which are also present in Croatia. The programs are shaped around three main topics: (1) Climate change (CC) impact and indicators; (2) Gene diversity and adaptive capacities of trees to CC; (3) Adding values to forest products and services (Strategy of Forest research institute development, 2012-2020, Hrvatski šumarski institut, 2012). The main components of each research program are presented in Fig. 1.

(1) To address **Climate change** the process of data collection for the purpose of monitoring activities is needed to be improved and harmonized especially in data collection, data storage, data analysis and interpretation of the results. The monitoring part of this program consists of monitoring of forest structure, standing biomass, growth, plant biodiversity, soil parameters together with a soil organic matter, hydrologic conditions etc. Important part of the monitoring activities is organized within the European level monitoring (UNECE ICP Forests network) at

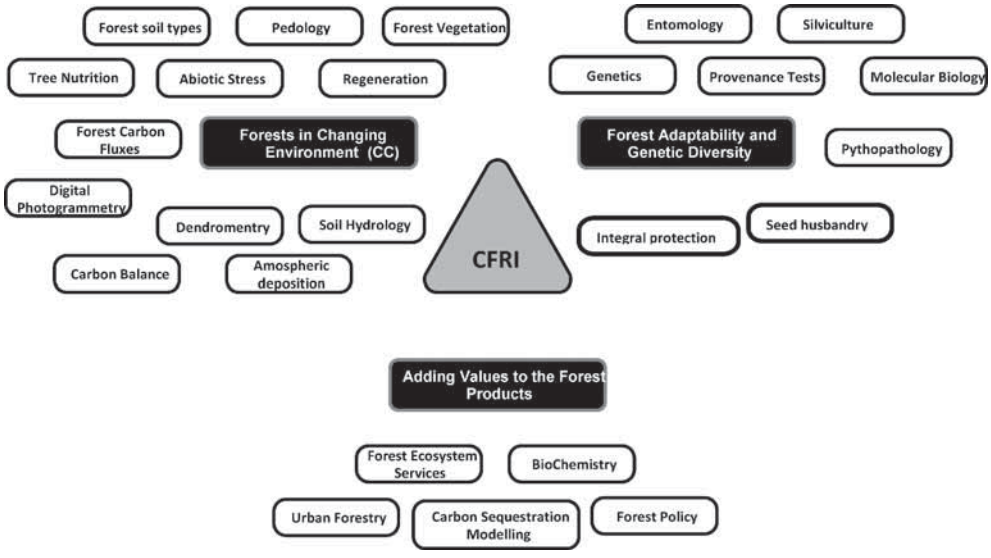


Fig. 1 – Main components of research programs of CFRI

level 1 plots (16 km x16 km grid) and on three plots on level 2, where intensive monitoring is performed (dry and wet deposition pollutant etc, (Vrbek et al. 2008, Jakovljević et al. 2009). In addition, sub-regional monitoring of groundwater resources in lowland forests has been set-up in the 1990s with sets of piezometric stations in continental part of Croatia. In the following period existing forest soil and hydrology monitoring network has to be renewed, modernized, extended and integrated with other monitoring activities.

One of the main research achievements regarding the carbon sequestration research was setting up of eddy covariance research station for measuring CO2 fluxes between Pedunculate oak forest and the atmosphere in 2007 (in close collaboration with University of Udine, Italy) and the assessment of ion fluxes from the atmosphere to the lowland forest ecosystem of Croatia following the UN-ECE ICP Forests methodologies in 2010 (in collaboration with CNR-ISE)

Carbon fluxes research at the Jastrebarsko Pedunculate oak forest consists of set of interconnected experiments aiming at describing different aspects of carbon cy-

Production of seedlings in the nursery HŠI

cling. Core of the experiment is eddy covariance flux station with meteorological station that provides data on Net Ecosystem Exchange (NEE) of CO2 at the boundary between forest canopies and atmosphere, using flux community standard online tool for gap-filling the data. Within, soil respiration measurements are conducted and state-of-the-art models are used relating soil respiration and environmental variables (soil temperature and humidity) to model fluxes from soil. This allows for integration of fluxes on daily, seasonal and annual level, and also comparison with Net Primary productivity (NPP) estimates from weekly measurements of tree trunk increments using dendrometer bands (Marjanović et al. 2009, Marjanović et al. 2011). Additional experiments of wood decomposition rates, root biomass estimates, dead wood and litter, etc. are conducted on regular intervals and the overall experiment has a tendency on covering all age classes of specific forest ecosystem using chronosequence approach. Furthermore, comprehensive database is compiled on the process of oak decline and dieback in the Spačva forest. Based on the analysis of these data, permanent experimental plots were established and decline process is monitored in detail, with special emphasis on the effects of the process on stand structure, as well as silvicultural options to mitigate these negative effects (Dubravac and Dekanić, 2009).

Monitoring of groundwater in the lowland soils is conducted using more than a hundred monitoring stations that provided up till now data series for 10 to 20 years (Pilaš et al. 2007). In recent years there has been a growing tendency to attribute the deterioration of crown condition of forest trees to various factors responsible for the health condition of forests. With recent lowering of the amount of sulphur and nitrogen compounds being deposited into forest ecosystems, the attention of the scientific community operating as a part of ICP Forests network has shifted towards the effects of climate change on forests (Potočić et al. 2008, Lorz et al. 2010). The International Co-operative Programme on Assessment and Monitoring of Air Pollution Effects on Forests (ICP Forests) under the Convention of Long-Range Transboundary Air Pollution (UN/ECE) and European Programme for the Intensive Monitoring of Forest Ecosystems Protection of Forests against Atmospheric Pollution (Regulation (EC) N° 3528/86 and its amendments) aim to conduct intensive and continuous monitoring of forest ecosystems in Europe as means of evaluating the damage caused by atmospheric pollution, acid loads, climate change and other factors influencing forest condition. In order to detect the influence of various stress factors the monitoring is carried out in the permanent plots located European countries, including in Croatia.

(2) Research on **Adaptive capacities and gene diversity** comprise three different but connected areas: A. Detecting of new invasive species in Croatia (Pernek et al 2009, Matošević et al. 2010), research of population dynamics of major forest pests in Croatia with standard and new developed methodologies, B. Use of existing databases, statistical methods and climate data for prognosis of population dynam-

ics of forest pests (Pernek et al. 2008), and C. Setting up various provenance tests: oak, beech and fir that allow us to collect information on the growth and development of domestic and foreign provenances (Ivanković et al. 2011; Ivanković et al. 2011)

With international collaboration we established international provenance tests with black pine and common beech (Ivanković et al. 2011). Furthermore, molecular analyses were conducted of Pedunculate oak along its distribution range in Balkan Peninsula (Ballian et al. 2010). Using sample research data from provenance trials various statistical analysis were conducted with the aim to determine the parameters of genetic diversity and genetic structure of species (Ivanković et al. 2011; Ivanković et al. 2011).

Assessment of genetic diversity of forest pests as a tool for measuring the biological potential and risk for the forest ecosystems is at the base of our research plans. In order to enable future collaborating with the industry and developing of innovative methods, establishing of high quality Laboratory of molecular-genetic testing in the forestry is planned. Upgrading of equipment for the laboratory would allow analyzing for molecular DNA regions of the forest tree species that are already developed for analysis of genetic diversity, genetic structure and resistance to intense of disease infection. The accreditation of the laboratory according to the international standard ISO/IEC 17025 would prove competence, thus the acquisition of new equipment is in the focus of this RG. To improve knowledge on plant pathogen diversity and their significance in the forest ecosystem including forest seeds, and to control the plant pathogens in changed forest ecosystems, modern stereomicroscope and environmental chamber are necessary as establishing a collection of plant pathogenic fungi for better understanding of pathogen variation and population dynamics.

(3) This Research component aims to identify a scientific basis for calculating **'soft forest values'** (e.g. improving landscape quality, soil protection, carbon sequestration, recreation possibilities, micro-climate amelioration etc.) to be found in the contribution from indirect goods and services such as recreation, tourism, health and the environment, that are under-utilized today. The research serves to transform the marketing of non-wood forest goods and services, helping to develop sustainable economic activities and also inform policymaking.

Determination of the most attractive and value adding applications and conversion routes for the miscellaneous, unique compounds present in herbs, foliage and other forest materials and non-wood goods will (*help in promotion*) promote conversion to high value-added **'green' specialty chemicals**. The extraction of the natural ingredients derived from the plant raw material with supercritical fluid extraction is a high-tech technology, recognized as the technology of the future in the pro-

duction of natural products. This process includes the use of low cost environmentally friendly solvents that are available on an unlimited scale both from renewable organic resources and from inorganic material, such as CO_2. The most promising areas will be identified for further product development. Commercial success will also involve developing efficient processes and specific product portfolios for specialty chemicals, which will be derived from various non-wood goods and herbs.

Awareness rising within the forest sector and related other sectors as well as among the general public and policy and decision makers is needed to **motivate and mobilize actions towards a resource efficient, low-carbon bio-economy**.

Addressing the challenges from policy perspective

The most intensive policy processes take place in area of **nature protection** but they gone much further from just define protected areas. Recent development is integration of nature protection measures into the management of the productive forest presenting a new challenge for forest policy in many countries for quite a long time. Although in most European countries, corresponding rules and guidelines have been established, implementation deficits prevail (Van Gossum et al 2008). This is also the case for Croatia and some other Eastern European Countries that have pushed progressive rules at the national level but lack the capacities and the comprehension to enforce them at the regional/management level. For many EU countries implementation of integrative nature protection in forests has also been a permanent topic.

Implementation of nature protection policy in Croatia has facing lack of capacities for integrative nature protection on side of protection and also for integrative forest management on forestry side. Although all state owned forests in Croatia are certified by Forest Stewardship Council (FSC) for more than 10 years (since 2002) there is still big gap in understanding of some of FSC standards related to protection of rare habitats and forests of high conservation values (HCV). Later new Forestry Act (Official Gazette, NN 140/05) prescribes as mandatory to incorporate Forest Europe Criteria and Indicators of sustainable forest management (C&I of SFM) in forest management plans (result of adopting the conclusions from 4th Ministerial conference on the Protection of Forest in Europe). One group of those criteria directly tackles nature protection. On top of that there was National ecological network preceding actual Natura 2000 proposal which comprise huge part of state

UŠP Senj island of Krk, the difference non-wooded and forested areas

and privately owned forests prescribing concrete measures of nature protection on specific site level.

We could say that policy and legislative framework exists but there is very little evidence on many of those measures on management or site level. But this is also situation in other EU countries and reason for that can be in nature of all those policies which work on basis of non-legally binding instruments, that means that implementation and even more important monitoring and reporting is up to each country. Recent development in direction to enable regular data collecting and monitoring of those criteria and indicators is seen in process of negotiation of legally binding instruments on forests (LBA) as continuation of all those processes (current situation is that negotiation is not finished and main questions are about position of council, definition of controlling systems and penalties). Croatia is actively involved in this process and is willing to accept rules when they will be defined. This will put another task in-front of forest administration and managers due to obligation to collect and report on evidence of implementation of C&I of SFM in forestry. The results of this project will directly help and be able to secure quality fulfilling of this task.

Under all those processes is goal to protect and preserve natural resources as main source of biodiversity, gene pool and many other services and products but most important role is mitigation of Climate change impacts which only health and stabile ecosystem can.

The recent literature on implementation of sustainable forest management emphasize (1) a flexible approach, (2) regional cooperation between forest owners, "intermediate institutions" at the mezzo or regional level and (3) participative procedures to build trust and social capital (Buttoud et al 2011) that might facilitate the acceptance of rules and the information flow between levels of government. Social network analysis had been applied to identify implementation networks that support the aforementioned factors and might thus facilitate the **implementation of nature protection in forests** (Primmer 2011; Paletto et al 2012). By building on recent **social network analysis** techniques (Friedkin and Johnsen, 2011), an increasing number of studies focus on implementation in **forest policy** evaluate the impact of different forms of network-coordination structures, particularly resource networks (Pfeffer and Salacnik, 1978). This allows identifying key actors and actors that act as intermediaries. Based on their results, they suggest that there is potential for stronger involvement of NGOs in the implementation process. Primmer (2011) evaluate different types of implementation networks to support the integration of biodiversity conservation into forestry in Finland. She concludes that such networks can foster learning if they allow informal coordination on top of formal contacts. Vuletić et al (2010a and 2012) explore learning possibilities in conflict management processes in Croatia and South East European countries focusing on conflicts between forestry and nature protection. The history and tradition as well as existing old structures and social networks were found by Vuletić et al (2010b) as main socio-economic conditions shaping forest policy development in SEE region.

Lovrić and Lovrić (2010) explore organizational structures in private forestry in Croatia. However, most of those networks focus on economic activities rather than on biodiversity protection, due to lacking incentives.

Instead of conclusion – Redefinition of role of science, research and institutes

Many governments express a growing need for having a science that is „usable ", which means that research results should be useful for practical application. US Congress was among the first to claim that only science useful for policy and decision makers could help reduce impact of climate change, and German Government

introduced the concept of departmental research as obligatory for all state departments.

In Croatia and some other South east European countries current strategies and laws addressing science and research see public research institutes as organizations with activities primarily oriented toward public interest, in a way that policy actors are provided with timely and adequate science-based information. One recent paper analyzed scientific and research activities of public research Institutes in Serbia and Croatia (Institute of Lowland Forestry and Environment (ILFE) – Serbia and Croatian Forest Research Institute (CFRI) – Croatia) to see whether, how and to what extent this institutions meet the needs of actors in policy processes, i.e. if it is in line with the requirements of „usable science". The Model of departmental research was used for these purposes. The very Model consists of following discourses: *research*, *integration*, scientific and practical *utilization,* whereas production lines connect them and stay for activities of research projects.

Results showed five types of production lines with more similarities than differences between two institutes:

(1) Preliminary research, starting in *integration* discourse, with the idea what will in the future be relevant for particular actor(s). However most activities remain in the *research* discourse, and foster results toward *scientific utilization*.

(2) Research stopped in the integration discourse. This production line also starts in the *integration* discourse, continues to the *research* discourse, where project proposal has been made, but its implementation gets stopped by the actors in political process, i.e. in the *integration* discourse.

(3) Research oriented toward practical utilization, where constant interaction between *research* and *integration* discourses, ends up in practical utilization of results.

(4) Research projects oriented toward scientific and practical utilization, with the main difference that results are meant not only for practice, but for the scientific community as well.

(5) Consulting activities of public institutes typically present projects which are usually entire within the *Integration* discourse with outputs in the *practical utilization* discourse (Stevanov et al 2013).

This research was able to present all projects of the both institutes with production lines on the Model of departmental research. The strong connection between Institutes and users of their expertise is illustrated with more than half of research pro-

jects having practical application. There are also important consulting activities going on which confirms importance of their role and quality of connection with the practice, policy makers and society.

The Model proves to be advantageous over existing evaluation methods, while it makes transparent all aspects of departmental research, which is useful for both users of science-based expertise and founders of these institutions. It also makes a solid base for optimization and quality management processes within institutions. Croatian public institutes have undergone process of reaccreditation which supposed to serve as starting point for redefinition of their role in society, and we find this research as very interesting in that sense to show more than research that taking place in daily institutes activities.

Croatian Forest Research Institute in a nutshell

CFRI (www.sumins.hr) is a public research institute owned by the Republic of Croatia. Its research objectives and tasks are part of the National Scientific-Research Program of the Republic of Croatia and Development Program of "Croatian Forests" ltd. Institute's principle goals are preservation of stability, productivity, biodiversity and genetic resources of forest ecosystems in Croatia based on the principles of sustainable forest management and natural rejuvenation. CFRI was founded in 1974.as the Forest research institute, Jastrebarsko by merging: Institute for forestry and wildlife research, Zagreb (founded in 1945), Yugoslav institute for conifers, Jastrebarsko (founded in 1960) and Institute for control of forest seeds, Rijeka (founded in 1959). In 2009 Forest research institute, Jastrebarsko changes its name into Croatian forest research institute.

Performing of research and services in the CFRI is executed by six scientific divisions; a service Department for Nursery Production, and three Regional Research Centers.

CFRI's research departments are:

Division for forest ecology which conducts research on forest ecology and soil science, and has key position in research and implementation of research projects, regional plans, silviculture, monitoring forest ecosystems. In recent years, great attention was given to the study of forest ecosystems and soil pollution with heavy metals and acidification of soils. Division is also in-charged for the implementation of International Co-operative Program on Assessment and Monitoring of Air Pollution Effects on Forests ICP Forests in the Republic of Croatia.

The field in the nursery with ornamental plants HŠI

Division for genetics, forest tree breeding and seed husbandry is engaged in provenance trials, establishment of clonal seed orchards, establishment of progeny tests and vegetative propagation, measuring of molecular differentiation and quality control of forest seeds.

Division for forest management and forestry economics is engaged in basic research and professional activities related to forest management and growth, balance and carbon cycle in terms of forest management, remote sensing ,forest economics and forest policy, evaluating multifunctional role of forests and other non-wood forest products and services.

Division for silviculture deals with the establishment and management of forest plantations where scientific studies are being continuously conducted for 45 years in international and domestic projects. The importance of this research is reflected in the conservation of biodiversity and ecological stability of forest plantations (especially in respect to forest fires in the coastal area, pests, windfall, avalanches, climate change, water regime, the impact of air pollution, etc.), maximum use of production potential of the habitat (timber, biomass) and general biodiversity of forests in Croatia.

Division for forest protection and game management is engaged in activities related to forest entomology, forest phytopathology, integrated forest protection and phitopharmacy. The focus of scientific research and technical expertise are pathogens and pests of trees and causes of forest degradation.

Division for international scientific cooperation in southeast Europe – EFISEE –coordinates and promotes research of products and services derived from forests and linking forestry in the region with the state-of-the-art forest research in Europe and the European Union. EFISEE is established to have impact on forestry research in South Eastern Europe with a focus on strengthening the existing potentials, expanding diversity and emphasizing the economic importance of forestry research.

Three **regional research centers** were established to connect research activity in the field of forestry with the local communities and experts in order to ensure sustainable management of forests and forest lands:
– Research Center for Urban and Private Forests in Varaždin;
– Research Center for Lowland Forests in Vinkovci.
– Research Center for Other Forest Goods and Services "Josip Ressel" in Pazin;

The quality of laboratory services at the CFRI is assured by implementing international quality standards ISO / IEC 17025, for that purpose the CFRI's Department for laboratory testing was formed. The Department for laboratory analysis provides its services in five laboratories:
– Laboratory for seed analysis;
– Laboratory for physical-chemical analysis;
– Laboratory for molecular-genetic analysis;
– Laboratory for entomological analysis;
– Laboratory for phytopathology analysis.

Highly skilled scientific staff as well as long tradition of scientific-research activity in the field of forestry is CFRI's major forces, along with the large number of young researchers who actively participate in national and international scientific initiatives and projects. Nowadays the CFRI employs 45 R&D and academic staff (whereas 24 PhD researchers, 4 MSc, 10 research assistants and 6 doctoral students), with 32 employees working either as administrative or technical staff.

Publishing activity of Croatian Forest Research Institute includes:
– Journal RADOVI (www.sumins.hr) (indexed in CABI and HRČAK);
– International scientific journal in the field of forestry science SEEFOR (www.seefor.eu), published in cooperation with renowned regional partners.

Publishing also includes monographs, bibliographies, symposium proceedings, reports, etc. CFRI's researchers act as reviewers in renowned scientific journals, their scientific work is regularly published and indexed in Web of Science, Scopus, CAB Direct databases.

With the objective to further implement scientific research in forestry ,maintain and promote sustainable management of forest ecosystems, raise awareness on the role of forests and importance of their preservation for future generations, by continuous monitoring and securing the continuity of ecosystem and environmental services – the role of CFRI as key national and leading regional scientific force in the field of forestry research is essential in the Republic of Croatia and part of our vision and mission.

References

1. A Strategic Research Agenda for Innovation, Competitiveness and Quality of Life, Forest-Based Sector Technology Platform, Brussels, 2006. http://www.efi.int/files/attachments/item_6_sra_ftp_final.pdf
2. Ballian, D., Ivanković, M., Gračan, J., Perić, S., Marjanović, H., Bobinac, M., Slade, D., 2010: Analysis of Pubescent Oak (*Quercus Pubescens* Willd.) by Means of Chloroplast DNA (cpDNA) in the Western Part of the Balkan Peninsula. *Acta Societatis Botanicorum Poloniae* 79 (3): 189-195.
3. Bodin, Ö., Crona, B., Ernston, H. (2006) "Social Networks in Natural Resource Management: What Is There to Learn from a Structural Perspective?" Ecology and Society 11 (2):r2. [online] URL: http://www.ecologyandsociety.org/vol11/iss2/resp2/.
4. Buttoud, G., Kouplevatskaya-Buttoud, I., Slee, B., Weiss, G. (2011) "Barriers to institutional learning and innovations in the forest sector in Europe: Markets, policies and stakeholders". Forest Policy and Economics 13(2): 124-131
5. Dubravac, T., Dekanić, S., 2009: Structure and dynamics of harvest of dead and declining trees of pedunculate oak in the stands of Spačva forest from 1996 to 2006 (in Croatian with English summary), *Šumarski list* 133 (7-8): 391-405.
6. EU Forest Action Plan, Commission of the European Communities, COM(2006) 302 final
7. EU Forest Strategy: for forests and the forest-based sector, COMMUNICATION FROM THE COMMISSION TO THE EUROPEAN PARLIAMENT, THE COUNCIL, THE EUROPEAN ECONOMIC AND SOCIAL COMMITTEE AND THE COMMITTEE OF THE REGIONS, Brussels, 20.9.2013, COM(2013) 659 final
8. EUROPE 2020 – A strategy for smart, sustainable and inclusive growth. European Commission, COM(2010)2020 final
9. Forestry Act, Official Gazette, NN 140/05,
10. Friedkin, N.E., Johnsen, E.C. 2011. Social Influence Network Theory: A Sociological Examination of Small Group Dynamics. New York: Cambridge University Press
11. Green Paper – 'From Challenges to Opportunities: Towards a Common Strategic Framework for EU research and innovation funding', European Commission, COM(2011)48

12. Intergovernmental Panel on Climate Change (IPCC) Fifth Assessment, Working Group 1 report, September 27th 2013

13. Ivanković, M., Popović, M., Bogdan, S., 2011: Acorn morphometric traits and seedling heights variation of Pedunculate oak (*Quercus robur* L.) from the seed stands in Croatia. **Šumarski list** 135 (13): 46-58.

14. Ivanković, M., Popović, M., Katičić, I., von Wuehlisch, G., Bogdan, S., 2011: Quantitative genetic variation of European beech (*Fagus sylvatica* L.) provenances from the Southeastern Europe, (in Croatian with English summary). **Šumarski list** 135 (13): 25-37.

15. Jakovljević, T., Berković, K., Tartari, G., Vrbek, B., Vorkapić-Furač, J., 2009: Atmospheric deposition in forest ecosystem of Europe and research of new methods for determination of phosphorus and ammonia within a framework of ICP forests. Šumarski list 133 (5-6): 267-278.

16. Law on forest reproductive material, Croatian Official Gazette 75/2009

17. Lead Market Initiative, Commission of the European Communities, COM(2007) 860 final

18. Lovrić. M., Lovrić, N., Vuletić, D., Martinić I. 2012. Development of the Concept and Implementation of National Forest Programmes With Reference to Croatia. SEEFOR 3(1), 49-60.

19. Lorz, C., Fuerst, C., Galić, Z., Matijašić, D., Podrazky, V., Potočić, N., Simončić, P., Strauch, M., Vacik, H., Makeschin, F., 2010: GIS-based probability assessment of natural hazards in forested landscapes of Central and South-Eastern Europe. *Environmental Management* 46(6): 920-930.

20. Management Plan (2006th-2015th), Hrvatske šume d.o.o., 2006: Šumskogospodarska osnova područja Republike Hrvatske. (ŠGO RH). Razdoblje važenja od 01.01.2006. do 31.12.2015. godine, Zagreb.

21. Marjanović, H., Alberti, G.,Balogh, J., Czóbel, S., Horváth, L., Jagodics, A., Nagy, Z., Ostrogović, M.Z., Peressotti, A., Führer, E., 2011: Measurements and estimations of biosphere-atmosphere exchange of greenhouse gases – Forests. *In*: L. Haszpra (*ed*), Atmospheric Greenhouse Gases: The Hungarian Perspective, Springer, pp. 65–90.

22. Marjanović, H., Ostrogović, M. Z., Alberti, G., Balenović, I., Paladinić, E., Indir, K., Peressotti, A., Vuletić, D., 2011: Carbon dynamics in younger stands of pedunculate oak during two vegetation periods (in Croatian with English summary). Šumarski list 135 (13): 59-73.

23. Matošević, D., Pernek, M., Hrašovec B., 2010: First record of oriental chestnut gall wasp (*Dryocosmus kuriphilus*) in Croatia, Šumarski list 134 (9-10): 497-502.

24. National Forest Policy and Strategy, Croatian Official Gazette 120/2003

25. Paladinić, E., Vuletić, D., Posavec, S., 2008: Review of the state of private forest ownership in the Republic of Croatia, Rad.Šumar.inst.Jastrebar. Vol.43, br.1. pp:1-74, Jastrebarsko.

26. Pernek, M., Avtzis, D. N., Hrašovec, B., Diminić, D., Wegensteiner, R., Stauffer, C., Cognato, A. I., 2008: Novel morphological and genetic markers for the discrimination of three European *Pityokteines* (*Coleoptera*: *Curculionidae*: *Scolytinae*) species. *Periodicum biologorum* 110 (4): 329-334.

27. Pernek, M., Hrašovec, B., Matošević, D., Pilaš, I., Kirisits, T., Moser, J. C., 2008: Phoretic mites of three bark beetles (*Pityokteines* spp.) on Silver fir. *Journal of Pest Science* 81 (1): 35-42.

28. Pfeffer J. Salacnik, G.R. 1978. The external control of organizations. New York: Harper & Row.

29. Pilaš, I., Lukić, N., Vrbek, B., Dubravac, T., Roth, V., 2007: The effect of groundwater decrease on short and long term variations of radial growth and dieback of mature Pedunculate oak (*Quercus robur* L.) stand. *Ekologia Bratislava* 26 (2): 122-131.

30. Pilaš, I., Feger, K. H., Vilhar U., Wahren A., 2010: Multidimensionality of Scales and Approaches for Forest-Water Interactions. *In*: Bredemeier, M., Cohen, S., Godbold, D., Lode, E., Pichler, V.,

Schleppi, P. (*eds*), Forest Management and the Water Cycle – An Ecosystem Based Approach. Ecological Studies 212, Heidelberg, London, New York: Springer, pp. 351-380.

31. Posavec S., D. Vuletić, 2004: Vision of forestry development in Croatia through national foret policy and strategy, (Vizija razvoja šumarstva kroz nacionalnu politiku i strategiju), Forest in Transition II, Chalanges in Strengthening of Capacities for Forest Policy Development in Countries with Economies in Transition, 211-222, United Nations University, Hong Kong.

32. Posavec, S., Čavlović, J., Šporčić, M. 2005: Small-scale Private Forest Management in Croatia, IUFRO International Symposium, Small-scale Forestry in a Changing Environment, Vilnius, Lithuania, pg. 159-165.

33. Potočić, N., Seletković, I., Ugarković, D., Jazbec, A., Mikac, S., 2008: The influence of climate properties on crown condition of Common beech (*Fagus sylvatica* L.) and Silver fir (*Abies alba* Mill.) on Velebit. *Periodicum biologorum* 110(2): 145-150.

34. State of Europe's Forests, FOREST EUROPE Ministerial Conference, 14-16 June 2011 in Oslo, Norway.

35. Stevanov, M., Böcher, M., Krott, M., Krajter, S., Vuletić, D., Orlović, S., (2013): The Reserach, Integration and Utilization (RIU) model as an analytical framework for the professionalization of departmental research organizations: Case studies of publicly funded forest research institutes in Serbia and Croatia, Forest Policy and Economics, http://dx.doi.org/10.1016/j.forpol.2013.03.006.

36. Strategy of Forest research institute development, 2012-2020, Hrvatski šumarski institut, 2012.

37. Strategy of physical planning in Republic of Croatia 1997, Ministarstvo prostornog uređenja, graditeljstva i stanovanja, Ministry of Environment Protection and Physical Planning, Zagreb.

38. Vrbek, B., Pilaš, I., Dubravac, T., Novotny, V., Dekanić, S., 2008: Chemical composition of throughfall and soil solution in Pedunculate oak and common hornbeam forests of Northwest Croatia. *Periodicum biologorum* 110 (3): 163-161.

39. Vuletić D., Stojanovska M., Avdibegović M., Nevenić R., Petrović N., Posavec S., Haska H., Peri L., Blagojević D. (2010a) Forest-Related Conflicts in the Sout-East European Region: Regional aspects and Case studies in Albania, Bosnia and Herzegovina, Croatia, Macedonia and Serbia, EFI Proceedings No. 58, pp: 117-130, EFI, Joensuu, Finland.

40. Vuletić, D., Avdibegović, M., Stojanovska, M., Nevenić, R., Haska, H., Posavec, S., Krajter, S., Peri, L., Marić. B. (2012): Contribution to the Understanding of Typology and Importance of Forest-related Conflicts in South East Europe Region, Periodicum Piologorum, vol,115, Zagreb.

41. Vuletić, D., Benko, M., Dubravac, T., Krajter, S., Novotny, V., Indir, K., Balenović, I., 2009: Review of nonmarket forest goods and services evaluation methods. *Periodicum biologorum* 111 (4): 515-521.

42. Vuletić, D., Posavec, S., Krajter, S., Paladinić, E., (2010c): Payments for evnironmental services (PES) in Croatia – public and professional perception and needs for adaptation, SEEFOR, Vol 1. No 2, pp: 61-67, Jastrebarsko.

43. Vuleti*ć,* D., Vacik, H., Poto*čić,* N., Krajter, S., F*ü*rst, C., Seletkovi*ć,* I., Makeschin, F., Simon*čič,* P., Lorz, C, Gali*ć,* Z., (2010b). How socio-economic conditions influence forest policy development in Central and South-Eastern Europe, Environmental management, Vol 46, 6, 931:940, DOI: 10.1007/s00267-010-9566-3. (IF 2010 = 1,503)

Part III

Novel Technologies

Marin Soljačić

MacArthur Fellow
Professor of Physics at MIT

DATE AND PLACE OF BIRTH

7th February 1974; Zagreb, Croatia

POSITIONS

- Professor; Physics Department, MIT, (2011-
- Associate Professor; Physics Department, MIT, (2010-2011)
- Correspondent Member; Croatian Academy of Engineering, (2009-
- MacArthur Fellow, (2008-
- Assistant Professor; Physics Department, MIT, (2005-2010)
- Principal Research Scientist; Research Laboratory of Electronics, MIT, (2003-2005)
- Pappalardo Fellow; Physics Department, MIT, (2000-2003)

SELECTED AWARDS

- ***Young Global Leader (YGL)*** of the World Economic Forum: 2011.
- ***MacArthur Fellowship Award***: 2008.
- ***SciAm50 Award*** of Scientific American for outstanding technological leadership (2007).
- ***TR35 Award*** of the Technology Review: one of top 35 innovators under the age of 35. (2006)
- ***Adolph Lomb Medal*** of the Optical Society of America for 2005.

EDUCATION

- Princeton University, PhD in physics, (1996-2000):
  - PhD thesis topic in Non-linear Optics with Prof. Mordechai Segev: "Instabilities in non-linear wave systems in optics" (academic year 1999/2000 I spent at Technion, Haifa, Israel)
  - MA thesis topic in Spin Glasses with Prof. Frank Wilczek: "A simple potential whose number of minima grows exponentially in the number of variables"
- MIT, undergraduate education, (1992-1996):
  - BSE in Physics
  - BSE in Electrical Engineering
  - Undergraduate thesis topic in Inflationary Cosmology with Prof. Lisa Randall, and Prof. Alan Guth: "Supernatural Inflation: Inflation from Supersymmetry with No (Very) Small Parameters"
- High-school: MIOC, (Zagreb, Croatia) (1988-1992)

CONTACT INFO

E-mail: soljacic@mit.eu
Snail-mail: Prof. Marin Soljacic, Room 6C-419, MIT, 77 Massachusetts Avenue, Cambridge, MA 02139, USA
Tel: +1-617-253-2467; Fax: +1-617-253-2562

Photonics and Modern Electro-Magnetics Group at MIT, Boston, US.

Prof. Marin Soljačić's research group at MIT

Technological advances of the past decade have enabled the control of the material structure at length-scales smaller than the wavelength of light. This enabled creation of new materials (e.g. photonic bandgap crystals, or various surface plasmon systems), whose optical properties are dramatically different than those of any naturally occurring material. For example, nanostructured materials which display diffraction-less propagation of light , exhibit negative

refraction, or support very slow propagation of light , have all been demonstrated. Our research interests are in exploring the new and exciting physical phenomena supported by such materials. Our work is roughly equally split between theoretical and experimental studies.

For some representative examples of this, please check out our work on one-way waveguides, plasmons in graphene, Dirac points in Photonic Crystals, a unique way of trapping light, or novel transparent displays.

Trip to Suzhou (China), May 2013.
Left to right: Jeongwon, Ling, Marin, and Wade.

The unique properties of optical nano-structured materials have already enabled a wide range of very important applications (e.g. in medicine, energy , telecommunications , defense, etc.) and are expected to do even more so in the future.

We are also interested in various topics in nonlinear optical physics. Maxwell's equations as presented in most undergraduate text books are linear. However, all materials in nature are nonlinear (including vacuum), and sure enough, at high light intensities, optical phenomena becomes nonlinear, displaying a wide range of rich and beautiful behavior. For example, almost every general non-linear dynamics phenomenon (e.g. solitons , pattern formation, fractals , etc.) can now be studied in optical material systems.

In addition, we are excited about the feasibility of wireless power transfer.

Principal Investigator: Prof. Marin Soljacic

Postdocs: Dr. Ling Lu, Dr. Ido Kaminer

Celebration of Lunar New Year, January 2012.
Left to right: Marin, Ognjen, Adrian, Veronika, David, Wade, Song, Zhiyu, Yichen, Wenjun, and Bo.

PhD students: Bo Zhen, Adrian Y. X. Yeng, Jeongwon Lee, Ognjen Ilic, Chia Wei Hsu, Yichen Shen, Scott Skirlo

Research Assistants: Emma Anquillare, Imbert Wang

Undergraduate students: Prashanth S Venkataram, Jared T. McKeon, Nicholas H Rivera,

Former postdocs and PhD Students: Dr. Veronika Rinnerbauer(senior post-doc at the Johannes Kepler University of Linz, Austria), Dr. Wenjun Qiu (quantitative researcher Citadel Investment Group), Dr. Song Liang Chua (scientist with DSO national laboratories, Singapore), Dr. Marinko Jablan (postdoc at University of Zagreb; PhD co-advised with Prof. Hrvoje Buljan), Dr. Dario Jukic (postdoc at University of Zagreb), Dr. Andre Kurs (scientist at WiTricity Corporation), Dr. David Duchesne, Prof. Rafif Hamam (Prof. at University of Dammam, Saudi Arabia), Prof. Jorge Bravo Abad (Prof. at Universidad Autonoma de Madrid, Spain), Prof. Yidong Chong (Prof. at NTU, Singapore), Prof. Peter Rakich (Prof. at Yale), Prof. Zheng Wang (Prof. at UT Austin)

Scientific Publications

Taken from: http://www.mit.edu/~soljacic/papers.html on 2/9/2014 3:10 PM

1. "Transparent displays enabled by resonant nanoparticle scattering" Chia Wei Hsu, Bo Zhen, Wenjun Qiu, Ofer Shapira, Brendan G. DeLacy, John D. Joannopoulos, and **Marin Soljacic**. ***Nature Communications***, Vol.7, 3152 (2014); doi:10.1038/ncomms4152. More material (here).
2. "Solar thermophotovoltaic energy conversion systems with two-dimensional tantalum photonic crystal absorbers and emitters" Youngsuk Nam, Yi Xiang Yeng, Andrej Lenert, Peter Bermel, Ivan Celanovic, **Marin Soljacic**, and Evelyn N.Wang. ***Solar Energy Materials & Solar Cells***, Vol.122, 287 (2014).
3. "Design of wide-angle selective absorbers/emitters with dielectric filled metallic photonic crystals for energy applications" J. Chou, Y. Yeng, A. Lenert, V. Rinnerbauer, I. Celanovic, **M. Soljacic**, E. Wang, and S. Kim. ***Optics Express*** Vol. 22, p.A144, (2014).
4. "Evolution of sputtered tungsten coatings at high temperature" Veronika Stelmakh, Veronika Rinnerbauer, John D. Joannopoulos, **Marin Soljacic**, Ivan Celanovic, and Jay J. Senkevich. ***J. Vac. Sci. Technol. A*** Vol. 31, p.061505, (2013).
5. "Stimulated Brillouin scattering in nanoscale silicon step-index waveguides: a general framework of selection rules and calculating SBS gain" Wenjun Qiu, Peter T. Rakich, Heedeuk Shin, Hui Dong, **Marin Soljacic**, and Zheng Wang. ***Optics Express*** Vol. 21, p.31402, (2013).
6. "Super-collimation with high frequency sensitivity in 2D photonic crystals induced by saddle-type van Hove singularities" Xulin Lin, Xiaogang Zhang, Liang Chen, **Marin Soljacic**, and Xunya Jiang. ***Optics Express*** Vol. 21, p.30140, (2013).

7. "Performance analysis of experimentally viable photonic crystal enhanced thermophotovoltaic systems" Y.X.Yeng, W.R.Chan, V.Rinnerbauer, J.D.Joannopoulos, **M.Soljacic**, and I.Celanovic. ***Optics Express*** Vol.21, p.A1035, (2013).

8. "High-temperature tantalum tungsten alloy photonic crystals: Stability, optical properties, and fabrication" V. Stelmakh, V. Rinnerbauer, R. D. Geil, P. R. Aimone, J. J. Senkevich, J. D. Joannopoulos, **M. Soljacic**, and I. Celanovic. ***Appl. Phys. Lett.*** Vol.103, 123903, (2013).

9. "Enabling enhanced emission and low-threshold lasing of organic molecules using special Fano resonances of macroscopic photonic crystals" B.Zhen, S-L.Chua, J.Lee, A.W.Rodriguez, X.Liang, S.G.Johnson, J.D.Joannopoulos, **M.Soljacic**, and O.Shapira. ***PNAS*** Vol.110, p.13711, (cover page), (2013).

10. "Layer-by-layer self-assembly of plexcitonic nanoparticles" Brendan G. DeLacy, Wenjun Qiu, **Marin Soljacic**, Chia Wei Hsu, Owen D. Miller, Steven G. Johnson, and John D. Joannopoulos. ***Optics Express*** Vol.21, p.19103, (2013). Also appeared as an ***Invited Paper in Virtual Journal for Biomedical Optics*** October 2013.

11. "Observation of trapped light within the radiation continuum" Chia Wei Hsu, Bo Zhen, Jeongwon Lee, Song-Liang Chua, Steven G. Johnson, J.D.Joannopoulos, and **Marin Soljacic**. ***Nature*** Vol.499, p.188, (2013).

12. "Bloch surface eigenstates within the radiation continuum" Chia Wei Hsu, Bo Zhen, Song-Liang Chua, Steven G. Johnson, J.D.Joannopoulos, and **Marin Soljacic**. ***Invited Article in Light: Science & Applications by Nature group*** **(2013) 2**, e84;doi:10.1038/lsa.2013.40.

13. "Plasmons in Graphene: Fundamental Properties and Potential Applications" Marinko Jablan, **Marin Soljacic**, and Hrvoje Buljan. ***Invited Review Paper in Proceedings of the IEEE*** Vol.101, p.1689, (2013).

14. "Damping of plasmons in graphene" Marinko Jablan, Hrvoje Buljan, and **Marin Soljacic**. *News&Views* in ***Nature Photonics*** Vol.7, p.346, (2013).

15. "High-temperature stability and selective thermal emission of polycrystalline tantalum photonic crystals" Veronika Rinnerbauer, Yi Xiang Yeng, Walker R. Chan, Jay J. Senkevich, John D. Joannopoulos, **Marin Soljacic** and Ivan Celanovic. ***Optics Express*** Vol.21, No.9 p.11482, (2013).

16. "Toward high-energy-density, high-efficiency, and moderate-temperature chip-scale thermophotovoltaics" W. R. Chan, P. Bermel, R. C. N. Pilawa- Podgurski, C. H. Marton, K. F. Jensen, J. J. Senkevich, J. D. Joannopoulos, **Marin Soljacic**, and Ivan Celanovic. ***PNAS*** Vol.110, No.14, p. 5309, (2013).

17. "Weyl points and line nodes in gyroid photonic crystals" Ling Lu, Liang Fu, John D. Joannopoulos, and **Marin Soljacic**. ***Nature Photonics***, Vol.7, 294 (cover page), (2013).

18. "Large-area fabrication of high aspect ratio tantalum photonic crystals for high-temperature selective emitters" Veronika Rinnerbauer, Sidy Ndao, Yi Xiang Yeng, Jay J. Senkevich, Klavs F. Jensen, John D. Joannopoulos, **Marin Soljacic** and Ivan Celanovic. ***Journal of Vacuum Science and Technology B*** Vol.31(1), 011802-1, (2013).

19. "Low emissivity high-temperature tantalum thin film coatings for silicon devices" Veronika Rinnerbauer, Jay J. Senkevich, John D. Joannopoulos, **Marin Soljacic** and Ivan Celanovic. ***Journal of Vacuum Science and Technology A*** Vol.31(1), 011501-1, (2013).

20. "Flat photonic surface bands pinned between Dirac points" Dario Jukic, Hrvoje Buljan, Dung-Hai Lee, John D. Joannopoulos, and **Marin Soljacic**. ***Optics Letters*** Vol.37, p.5262, (2012).

21. "Three-dimensional photonic crystals by large-area membrane stacking" Ling Lu, Lin Lee Cheong, Henry I. Smith, Steven G. Johnson, John D. Joannopoulos, and **Marin Soljacic**. ***Optics Letters*** Vol.37, p.4726, (2012).

22. “Recent developments in high-temperature photonic crystals for energy conversion” Veronika Rinnerbauer, Sidy Ndao, Yi Xiang Yeng, Walker R. Chan, Jay J. Senkevich, John D. Joannopoulos, **Marin Soljacic** and Ivan Celanovic. ***Invited Review Article in Energy & Environmental Science*** Vol.5, 8815, (2012).

23. “Observation and Differentiation of Unique High-Q Optical Resonances Near Zero Wave Vector in Macroscopic Photonic Crystal Slabs” Jeongwon Lee, Bo Zhen, Song-Liang Chua, Wenjun Qiu, John D. Joannopoulos, **Marin Soljacic**, and Ofer Shapira. ***Phys. Rev. Lett.*** Vol.109, 067401, (2012).

24. “Gyrotropic response in the absence of a bias field” Zhiyu Wang, Zheng Wang, Jingyu Wang, Bin Zhang, Jiangtao Huangfu, John D. Joannopoulos, **Marin Soljacic**, and Lixin Ran. ***PNAS*** Vol.109, No.33, p. 13194, (2012).

25. “Optimization of broadband optical response of multilayer nanospheres” Wenjun Qiu, Brendan G. DeLacy, Steven G. Johnson, John D. Joannopoulos, and **Marin Soljacic**. ***Optics Express*** Vol.20, No.16 p.18494, (2012).

26. “Enabling single-mode behavior over large areas with photonic Dirac cones” Jorge Bravo-Abad, John D. Joannopoulos, and **Marin Soljacic**. ***PNAS*** Vol.109, No.25, p. 9761, (2012).

27. “Waveguiding at the Edge of a Three-Dimensional Photonic Crystal” Ling Lu, John D. Joannopoulos, and **Marin Soljacic**. ***Phys. Rev. Lett.*** Vol.108, 243901, (2012).

28. “Fractal optics and beyond” Mordechai Segev, **Marin Soljacic** and John Dudley. *Commentary* in ***Nature Photonics*** Vol.6, 209, (2012).

29. “Near-field thermal radiation transfer controlled by plasmons in graphene” Ognjen Ilic, Marinko Jablan, John D. Joannopoulos, Ivan Celanovic, Hrvoje Buljan, and **Marin Soljacic**. ***Phys. Rev. B*** Vol.85, 155422, (2012).

30. “Overcoming the black body limit in plasmonic and graphene near-field thermophotovoltaic systems” Ognjen Ilic, Marinko Jablan, John D. Joannopoulos, Ivan Celanovic, and **Marin Soljacic**. ***Optics Express*** Vol.20, No.S3 p.A366, (2012).

31. “Enabling high-temperature nanophotonics for energy applications” Yi Xiang Yeng, Michael Ghebrebrhan, Peter Bermel, Walker R. Chan, John D. Joannopoulos, **Marin Soljacic**, and Ivan Celanovic. ***PNAS*** Vol.109, No.7, p. 2280, (2012).

32. “Fabrication of two-dimensional tungsten photonic crystals for high-temperature applications” M. Araghchini, Y. X. Yeng, N. Jovanovic, P. Bermel, L. A. Kolodziejski, **M. Soljacic**, I. Celanovic, and J. D. Joannopoulos. ***J. Vac. Sci. Technol. B*** Vol.29, No.6, p.061402, (2011).

33. “Broadband circulators based on directional coupling of one-way waveguides” Wenjun Qiu, Zheng Wang, and **Marin Soljacic**. ***Invited Paper in a Focus Issue of Optics Express*** Vol.19, No.22, p.22248, (2011).

34. “Tailoring photonic metamaterial resonances for thermal radiation” Peter Bermel, Michael Ghebrebrhan, Michael Harradon, Yi Xiang Yeng, Ivan Celanovic, John D. Joannopoulos, and **Marin Soljacic**. ***Nanoscale Research Letters*** Vol.6, p.549, (2011).

35. “Frequency-Selective Near-Field Radiative Heat Transfer between Photonic Crystal Slabs: A Computational Approach for Arbitrary Geometries and Materials” Alejandro W. Rodriguez, Ognjen Ilic, Peter Bermel, Ivan Celanovic, John D. Joannopoulos, **Marin Soljacic**, and Steven G. Johnson. ***Phys. Rev. Lett.*** Vol.107, 114302, (2011).

36. “Thermophotovoltaic power conversion systems: current performance and future potential” Ivan Celanovic, Peter Bermel, and **Marin Soljacic**. ***Invited Review Article in Oyo Buturi (Japan Society of Applied Physics)*** Vol.80, No.8, p.687, (2011).

37. “Abrupt coupling between strongly dissimilar waveguides with 100% transmission” Andre Kurs, John D. Joannopoulos, **Marin Soljacic**, Steven G. Johnson. ***Optics Express*** Vol.19, No.14, p. 13714, (2011).

38. “Transverse electric plasmons in bilayer graphene” Marinko Jablan, Hrvoje Buljan, **Marin Soljacic**. ***Optics Express*** Vol.19, No.12, p. 11236, (2011).

39. “Unconventional plasmon-phonon coupling in graphene” Marinko Jablan, **Marin Soljacic**, and Hrvoje Buljan. ***Phys. Rev. B Rapid Comm.*** Vol.83, 161409(R), (2011).

40. “Spatio-temporal theory of lasing action in optically-pumped rotationally excited molecular gases” Song-Liang Chua, Christine A. Caccamise, Dane J. Phillips, John D. Joannopoulos, **Marin Soljacic**, Henry O. Everitt, and Jorge Bravo-Abad. ***Optics Express*** Vol.19, No.8, p. 7513, (2011).

41. “Degenerate four-wave mixing in triply resonant Kerr cavities” David M. Ramirez, Alejandro W. Rodriguez, Hila Hashemi, J. D. Joannopoulos, **Marin Soljacic**, and Steven G. Johnson. ***Phys. Rev. A*** Vol.83, 033834, (2011).

42. “Design and global optimization of high-efficiency solar thermal systems with tungsten cermets” David Chester, Peter Bermel, John D. Joannopoulos, **Marin Soljacic**, and Ivan Celanovic. ***Optics Express*** Vol.19, No.S3, p. A245, (2011).

43. “Angular photonic band gap” Rafif E. Hamam, Ivan Celanovic, **Marin Soljacic**. ***Phys. Rev. A*** Vol.83, 035806, (2011).

44. “Tailoring thermal emission via Q matching of photonic crystal resonances” Michael Ghebrebrhan, Peter Bermel, Yi X. Yeng, Ivan Celanovic, **Marin Soljacic**, and John D. Joannopoulos. ***Phys. Rev. A*** Vol.83, 033810, (2011).

45. “Low-threshold lasing action in photonic crystal slabs enabled by Fano resonances” Song-Liang Chua, Yidong Chong, A. Douglas Stone, **Marin Soljacic**, and Jorge Bravo-Abad. ***Optics Express*** Vol.19, No.2, p. 1539, (2011).

46. “Design and global optimization of high-efficiency thermophotovoltaic systems” Peter Bermel, Michael Ghebrebrhan, Walker Chan, Yi Xiang Yeng, Mohammad Araghchini, Rafif Hamam, Christopher H. Marton, Klavs F. Jensen, **Marin Soljacic**, John D. Joannopoulos, Steven G. Johnson, and Ivan Celanovic. ***Optics Express*** Vol.18, No.103, p. A314, (2010).

47. “Efficient low-power terahertz generation via on-chip triply-resonant nonlinear frequency mixing” Jorge Bravo-Abad, Alejandro W. Rodriguez, J. D. Joannopoulos, Peter T. Rakich, Steven G. Johnson, and **Marin Soljacic**. ***Appl. Phys. Lett.*** Vol.96, 101110, (2010).

48. “Light scattering from anisotropic particles: propagation, localization, and nonlinearity” Chengwei Qiu, Lei Gao, John D. Joannopoulos, and **Marin Soljacic**. ***Laser & Photonics Reviews*** Vol.4, No.2, p.268, (2010).

49. “Simultaneous mid-range power transfer to multiple devices” Andre Kurs, Robert Moffatt, and **Marin Soljacic**. ***Appl. Phys. Lett.*** Vol.96, 044102, (2010).

50. “Plasmonics in graphene at infrared frequencies” Marinko Jablan, Hrvoje Buljan, and **Marin Soljacic**. ***Phys. Rev. B*** Vol.80, 245435, (2009). Also appeared as an ***Invited Paper in Virtual Journal of Nanoscale Science and Technology*** January 2010.

51. “Observation of unidirectional backscattering-immune topological electromagnetic states” Zheng Wang, Yidong Chong, J.D.Joannopoulos, and **Marin Soljacic**. ***Nature*** Vol.461, p.772, (2009).

52. “Plasmonic-Dielectric Systems for High-Order Dispersionless Slow or Stopped Subwavelength Light” Aristeidis Karalis, J.D.Joannopoulos, and **Marin Soljacic**. ***Phys. Rev. Lett.*** Vol.103, 043906, (2009). Also appeared as an ***Invited Paper in Virtual Journal of Nanoscale Science and Technology*** July 2009.

53. “Ultrafast photodetection in an all-silicon chip enabled by two-photon absorption” J. Bravo-Abad, E. P. Ippen, and **Marin Soljacic**. ***Appl. Phys. Lett.*** Vol.94, 241103, (2009). Also appeared as an ***Invited Paper in Virtual Journal of Ultrafast Science*** July 2009.

54. “Efficient weakly-radiative wireless energy transfer: An EIT-like approach” Rafif E. Hamam, Aristeidis Karalis, J.D. Joannopoulos, and **Marin Soljacic**. ***Annals of Physics*** Vol.324, 1783, (2009).

55. “Broadband super-collimation in a hybrid photonic crystal structure” Rafif E. Hamam, Mihai Ibanescu, Steven G. Johnson, J. D. Joannopoulos, and **Marin Soljacic**. ***Optics Express*** Vol.17, 8109, (2009).

56. “Nonlinear harmonic generation and devices in doubly resonant Kerr cavities” Hila Hashemi, Alejandro W. Rodriguez, J. D. Joannopoulos, **Marin Soljacic**, and Steven G. Johnson. ***Phys. Rev. A*** Vol.79, 013812, (2009).

57. “Supercollimation in photonic crystals composed of silicon rods” Ta-Ming Shih, Andre Kurs, Marcus Dahlem, Gale Petrich, **Marin Soljacic**, Erich Ippen, Leslie Kolodziejski, Katherine Hall, and Morris Kesler. ***Appl. Phys. Lett.*** Vol.93, 131111, (2008). Also appeared as an ***Invited Paper in Virtual Journal of Nanoscale Science & Technology*** 20th October 2008.

58. “Effective theory of quadratic degeneracies” Y. D. Chong, Xiao-Gang Wen, and **Marin Soljacic**. ***Phys. Rev. B*** Vol.77, 235125, (2008).

59. “Purcell effect in nonlinear photonic structures: A coupled mode theory analysis” Rafif Hamam, Mihai Ibanescu, Evan J. Reed, Peter Bermel, Steven G. Johnson, Erich Ippen, John D. Joannopoulos, and **Marin Soljacic**. ***Optics Express*** Vol.16, 12523, (2008).

60. “Nonlinear photonic crystals near the supercollimation point” Zhiyong Xu, Bjorn Maes, Xunya Jiang, John D. Joannopoulos, Lluis Torner, and **Marin Soljacic**. ***Optics Letters***, Vol.33, 1762, (2008).

61. “Efficient mid-IR spectral generation via spontaneous fifth-order cascaded-Raman amplification in silica fibers” Peter T. Rakich, Yoel Fink, and **Marin Soljacic**. ***Optics Letters***, Vol.33, 1690, (2008).

62. “A Unified Picture of Laser Physics” Jorge Bravo-Abad, and **Marin Soljacic**. *Perspectives* in ***Science*** Vol.320, 623, (2008).

63. “Reflection-Free One-Way Edge Modes in a Gyromagnetic Photonic Crystal” Zheng Wang, Y.D.Chong, John D.Joannopoulos, and **Marin Soljacic**. ***Phys. Rev. Lett.*** Vol.100, 013905, (2008). Also appeared as an ***Invited Paper in Virtual Journal of Nanoscale Science & Technology*** 21st January 2008.

64. “Dark-state polaritons in single- and double-Lambda media” Y.D.Chong, and **Marin Soljacic**. ***Phys. Rev. A*** Vol.77, 013823, (2008).

65. “Efficient wireless non-radiative mid-range energy transfer” Aristeidis Karalis, John D.Joannopoulos, and **Marin Soljacic**. ***Annals of Physics*** Vol.323, 34, (2008).

66. “Trapping, corralling and spectral bonding of optical resonances through optically induced potentials” Peter T. Rakich, Milos A. Popovic, **Marin Soljacic** and Erich P. Ippen. ***Nature Photonics***, Vol.1, 658 (***cover page***), (2007).

67. “Enhanced nonlinear optics in photonic-crystal microcavities” Jorge Bravo-Abad, Alejandro Rodriguez, Peter Bermel, Steven G. Johnson, John D. Joannopoulos, and **Marin Soljacic**. ***Invited Paper in Optics Express*** Vol.15, 16161, (2007).

68. “Photonic Crystals go Dynamic” Jorge Bravo-Abad, and **Marin Soljacic**. *News&Views* in ***Nature Materials*** Vol.6, 799, (2007).

69. "Modeling Nonlinear Optical Phenomena in Nanophotonics" Jorge Bravo-Abad, Shanhui Fan, Steven G. Johnson, John D. Joannopoulos, and **Marin Soljacic**. ***Invited Paper in IEEE Journal of Lightwave Technology*** Vol.25, 2539, (2007).

70. "Molecular dynamics simulations of coherent optical photon emission from shock waves in crystals" Evan J.Reed, **Marin Soljacic**, Richard Gee, and J.D.Joannopoulos. ***Phys. Rev. B*** Vol.75, 174302, (2007).

71. "Distinguishing zero-group-velocity modes in photonic crystals" M.Ghebrebrhan, M.Ibanescu, Steven G.Johnson, **Marin Soljacic**, and J.D.Joannopoulos. ***Phys. Rev. A*** Vol.76, 063810, (2007).

72. "The nonlinear effect from the interplay between the nonlinearity and the supercollimation of photonic crystal" Xunya Jiang, Chuanhong Zhou, Xiaofang Yu, Shanhui Fan, **Marin Soljacic** and J. D. Joannopoulos. ***Appl. Phys. Lett.*** Vol.91, 031105, (2007).

73. "Tailoring Optical Nonlinearities via the Purcell Effect" Peter Bermel, Alejandro Rodriguez, John D. Joannopoulos, and **Marin Soljacic**. ***Phys. Rev. Lett.*** Vol.99, 053601, (2007). Also appeared as an ***Invited Paper in Virtual Journal of Nanoscale Science & Technology*** 20th August 2007.

74. "Wireless Power Transfer via Strongly Coupled Magnetic Resonances" Andre Kurs, Aristeidis Karalis, Robert Moffatt, J.D.Joannopoulos, Peter Fisher, and **Marin Soljacic**. ***Science*** Vol.317, 83, (2007). Also appeared in ***Science Express***, an advance on-line publication of ***Science*** 7th June 2007.

75. "Quantum theory of a resonant photonic crystal" Y. D. Chong, David E. Pritchard, and **Marin Soljacic**. ***Phys. Rev. B*** Vol.75, 235124, (2007).

76. "Chi(2) and Chi(3) harmonic generation at a critical power in inhomogeneous doubly resonant cavities" Alejandro Rodriguez, **Marin Soljacic**, J. D. Joannopoulos, and Steven G. Johnson. ***Optics Express*** Vol.15, 7303, (2007).

77. "Terahertz radiation from shocked materials" Evan J. Reed, Michael R. Armstrong, Kiyong Kim, **Marin Soljacic**, Richard Gee, James H. Glownia, and John D. Joannopoulos. ***Invited review article in Materials Today***, Vol.10, 44, (2007).

78. "Coupled-mode theory for general free-space resonant scattering of waves" Rafif E. Hamam, Aristeidis Karalis, J. D. Joannopoulos, and **Marin Soljacic**. ***Phys. Rev. A*** Vol.75, 053801, (2007).

79. "Maxwell equation simulations of coherent optical photon emission from shock waves in crystals" Evan J. Reed, **Marin Soljacic**, and J.D.Joannopoulos. ***Phys. Rev. E*** Vol.75, 056611, (2007).

80. "Extraordinary optical transmission through subwavelength holes in a polaritonic silicon dioxide film" Dye-Zone A. Chen, Rafif Hamam, **Marin Soljacic**, J.D.Joannopoulos, and Gang Chen. ***Appl. Phys. Lett.*** Vol.90, 181921, (2007).

81. "Emulating one-dimensional resonant Q-matching behavior in a two-dimensional system via Fano resonances" David L. C. Chan, Ivan Celanovic, J. D. Joannopoulos, and **Marin Soljacic**. ***Phys. Rev. A*** Vol.74, 064901, (2006). Also appeared as an ***Invited Paper in Virtual Journal of Nanoscale Science & Technology*** 15th January 2007.

82. "Photonic crystal optical waveguides for on-chip Bose-Einstein condensates" J. Bravo-Abad, M. Ibanescu, J. D. Joannopoulos, and **Marin Soljacic**. ***Phys. Rev. A*** Vol.74, 053619, (2006). Also appeared as an ***Invited Paper in Virtual Journal of Nanoscale Science & Technology*** 4th December 2006.

83. "Switching through symmetry breaking in coupled nonlinear micro-cavities" Bjorn Maes, **Marin Soljacic**, John D. Joannopoulos, Peter Bienstman, Roel Baets, Simon-Pierre Gorza, and Marc Haelterman. ***Optics Express*** Vol.14, 10678, (2006).

84. "Single-photon all-optical switching using waveguide-cavity quantum electrodynamics" Peter Bermel, Alejandro Rodriguez, Steven G. Johnson, John D. Joannopoulos, and **Marin Soljacic**. ***Phys. Rev. A*** Vol.74, 043818, (2006).

85. “Direct calculation of thermal emission for three-dimensionally periodic photonic crystal slabs” David L.C. Chan, **Marin Soljacic**, and J.D.Joannopoulos. ***Phys. Rev. E*** Vol.74, 036615, (2006). Also appeared as an ***Invited Paper in Virtual Journal of Nanoscale Science & Technology*** 2nd October 2006.

86. “Thermal emission and design in 2D-periodic metallic photonic crystal slabs” David L.C. Chan, **Marin Soljacic**, and J.D.Joannopoulos. ***Optics Express*** Vol.14, 8785, (2006).

87. “Thermal emission and design in one-dimensional periodic metallic photonic crystal slabs” David L.C. Chan, **Marin Soljacic**, and J.D.Joannopoulos. ***Phys. Rev. E*** Vol.74, 016609, (2006).

88. “Comment on “Explanation of the Inverse Doppler Effect Observed in Nonlinear Transmission Lines”” Evan Reed, **Marin Soljacic**, and John D. Joannopoulos. Technical Comment in ***Phys. Rev. Lett.***, Vol.96, 069402, (2006).

89. “Achieving centimetre scale super collimation in a large area 2D photonic crystal” Peter T. Rakich, Marcus S. Dahlem, Sheila Tandon, Mihai Ibanescu, **Marin Soljacic**, Gale S. Petrich, John D. Joannopoulos, Leslie A. Kolodziejski and Erich P. Ippen. ***Nature Materials***, Vol.5, 93 (***cover page***), (2006).

90. “Coherent optical photons from shock waves in crystals” Evan Reed, **Marin Soljacic**, Richard Gee, and J.D.Joannopoulos. ***Phys. Rev. Lett.***, Vol.96, 013904, (2006).

91. “Reversed and anomalous Doppler effects in photonic crystals and other time-dependent periodic media” Evan Reed, **Marin Soljacic**, Mihai Ibanescu, and J.D.Joannopoulos. ***Invited paper in Journal of Computer-Aided Materials Design***, Vol. 12, 1, (2005).

92. “Surface-Plasmon-assisted guiding of broadband slow and subwavelength light in air” Aristeidis Karalis, E.Lidorikis, Mihai Ibanescu, J.D.Joannopoulos, and **Marin Soljacic**. ***Phys. Rev. Lett.*** Vol.95, 063901, (2005).

93. “Roughness losses and volume-current methods in photonic-crystal waveguides” Steven G. Johnson, M.L.Povinelli, **Marin Soljacic**, Aristeidis Karalis, S.Jacobs, and J.D.Joannopoulos. ***Invited paper in Applied Physics B***, Vol.81, 283, (2005).

94. “The Superprism Effect using Large Area 2D-Periodic Photonic Crystal Slabs” S.N.Tandon, **Marin Soljacic**, G.S.Petrich, J.D.Joannopoulos and L.A.Kolodziejski. ***Photonics and Nanostructures - Fundamentals and Applications***, Vol.3, 10, (2005).

95. “Ultra Low-Power All-Optical Switching” **Marin Soljacic**, Elefterios Lidorikis, J.D.Joannopoulos, and Lene Vestergaard Hau. ***Appl. Phys. Lett.***, Vol.86, 171101, (2005). Also appeared as an ***Invited Paper in Virtual Journal of Nanoscale Science & Technology*** 2nd May 2005, and as an ***Invited Paper in Virtual Journal of Ultrafast Science*** May 2005.

96. “Enhancement of microcavity lifetimes using highly dispersive materials” **Marin Soljacic**, Elefterios Lidorikis, Lene Vestergaard Hau and J.D.Joannopoulos. ***Phys. Rev. E.***, Vol.71, 026602, (2005). Also appeared as an ***Invited Paper in Virtual Journal of Nanoscale Science & Technology*** 21st February 2005.

97. “Microcavities with highly dispersive materials” **Marin Soljacic**, Elefterios Lidorikis, J.D.Joannopoulos, and Lene Vestergaard Hau. ***Invited article in IEEE LEOS Newsletter***, Vol.18, No.5, 12, October (2004).

98. “Third order nonlinearities in Ge-As-Se-based glasses for telecommunications applications” Juliet T. Gopinath, **Marin Soljacic**, Erich P. Ippen, Vladimir N. Fuflyigin, Wesley A. King, and Max Shurgalin. ***Journal of Applied Physics***, Vol.96, 6931, (2004). Also appeared as an ***Invited Paper in Virtual Journal of Ultrafast Science*** December 2004.

99. “Comment on “Observation of the Inverse Doppler Effect”” Evan Reed, **Marin Soljacic**, Mihai Ibanescu, and J.D.Joannopoulos. Technical Comment in ***Science***, Vol.305, 778b, (2004).

100. "Optical Bistability and Cutoff Solitons in Photonic Bandgap Fibers" **Marin Soljacic**, Elefterios Lidorikis, Mihai Ibanescu, Steven G. Johnson, J.D.Joannopoulos, and Yoel Fink. ***Invited paper in Optics Express***, Vol.12, No.8, 1518, (2004).

101. "Enhancement of non-linear effects using photonic crystals" **Marin Soljacic**, and J.D. Joannopoulos. ***Invited review article in Nature Materials***, Vol.3, 211, (2004).

102. "Effect of a photonic band gap on scattering from waveguide disorder" M. L. Povinelli, Steven G. Johnson, Elefterios Lidorikis, J. D. Joannopoulos, and **Marin Soljacic**. ***Appl. Phys. Lett.***, Vol.84, 3639, (2004). Also appeared as an ***Invited Paper in Virtual Journal of Nanoscale Science & Technology*** 10th May 2004.

103. "Cutoff solitons in axially uniform systems" Elefterios Lidorikis, **Marin Soljacic**, Mihai Ibanescu, Yoel Fink, and J.D.Joannopoulos. ***Optics Letters***, Vol.29, 851, (2004).

104. "Superprism effect based on phase velocities" Chiyan Luo, **Marin Soljacic**, and J.D.Joannopoulos. ***Optics Letters***, Vol.29, 745, (2004).

105. "Polychromatic partially spatially inoherent solitons in non-instantaneous Kerr nonlinear medium" Hrvoje Buljan, Tal Schwartz, Mordechai Segev, **Marin Soljacic**, and Demetrios Christodoulides. ***JOSA B***, Vol.21, 397, (2004).

106. "Pattern formation in optical cavities with incoherent light" Tal Carmon, Hrvoje Buljan, **Marin Soljacic**, Mordechai Segev. ***Optics & Photonics News*** (***Optics in 2003***: special end-of-year issue), 40, December (2003).

107. "All-Optical Transistor Action With Bistable Switching in a Photonic Crystal Cross-Waveguide Geometry" Mehmet Fatih Yanik, Shanhui Fan, **Marin Soljacic**, and J.D.Joannopoulos. ***Optics Letters***, Vol.28, 2506, (2003).

108. "Incoherent white light solitons in logarithmically saturable noninstantaneous nonlinear media" Hrvoje Buljan, Antonio Siber, **Marin Soljacic**, Tal Schwartz, Mordechai Segev, and Demetrios Christodoulides. ***Phys. Rev. E***, Vol.68, 036607, (2003).

109. "High-Contrast All-Optical Bistable Switching in Photonic Crystal Microcavities" Mehmet Fatih Yanik, Shanhui Fan, and **Marin Soljacic**. ***Appl. Phys. Lett.***, Vol.83, 2739, (2003).

110. "Color of shock waves in photonic crystals" Evan Reed, **Marin Soljacic**, and J.D.Joannopoulos. ***Phys. Rev. Lett.*** Vol.90, 203904, (2003). Also appeared as an ***Invited Paper in Virtual Journal of Nanoscale Science & Technology*** 9th June 2003.

111. "Reversed Doppler effect in photonic crystals" Evan Reed, **Marin Soljacic**, and J.D.Joannopoulos. ***Phys. Rev. Lett.***, Vol.91, 133901 (***cover page***), (2003). Also appeared as an ***Invited Paper in Virtual Journal of Nanoscale Science & Technology*** 6th October 2003.

112. "Optical Bistability in Axially Modulated OmniGuide Fibers" **Marin Soljacic**, Mihai Ibanescu, Steven G. Johnson, J.D.Joannopoulos, and Yoel Fink. ***Optics Letters*** Vol.28, 516, (2003).

113. "Non-linear Photonic Crystal Micro-Devices for Optical Integration" **Marin Soljacic**, Chiyan Luo, J.D.Joannopoulos, and Shanhui Fan. ***Optics Letters*** Vol.28, 637, (2003).

114. "Analysis of Mode Structure in Hollow Dielectric Waveguide Fibers" Mihai Ibanescu, Steven G. Johnson, **Marin Soljacic**, J.D.Joannopoulos, Yoel Fink, Ori Weisberg, Torkel D. Engeness, Steven A. Jacobs, and M.Skorobogatiy. ***Phys. Rev. E*** Vol.67, 046608, (2003).

115. "Cavity pattern formation with incoherent light" Hrvoje Buljan, **Marin Soljacic**, Tal Carmon, and Mordechai Segev. ***Phys. Rev. E.*** Vol.68, 016616, (2003).

116. "White light solitons" Hrvoje Buljan, Mordechai Segev, **Marin Soljacic**, Nikolaos K. Efremidis, and Demetrios N. Christodoulides. ***Optics Letters*** Vol.28, 1239, (2003).

117. “Collisions of two solitons in an arbitrary number of coupled nonlinear Schrodinger equations” **Marin Soljacic**, Ken Steiglitz, Suzanne M. Sears, Mordechai Segev, Mariusz H. Jakubowski, and Richard Squier. ***Phys. Rev. Lett.*** Vol.90, 254102, (2003).

118. “Analysis of general geometric scaling perturbations in a transmitting waveguide: fundamental connection between polarization-mode dispersion and group-velocity dispersion” Maksim Skorobogatiy, Mihai Ibanescu, Steven G. Johnson, Ori Weisberg, Torkel D. Engeness, **Marin Soljacic**, Steven A. Jacobs, and Yoel Fink. ***JOSA B*** Vol.19, 2867, (2002).

119. “Optimal bistable switching in nonlinear photonic crystals” **Marin Soljacic**, Mihai Ibanescu, Steven G. Johnson, Yoel Fink, and J.D.Joannopoulos. ***Phys. Rev. E Rapid Comm.*** Vol.66, 055601(R), (2002). Also appeared as an ***Invited Paper in Virtual Journal of Nanoscale Science & Technology*** 25th November 2002.

120. “Pattern formation in a cavity longer than the coherence length of the light in it” Tal Carmon, **Marin Soljacic**, and Mordechai Segev. ***Phys. Rev. Lett.*** Vol.89, 183902, (2002).

121. “Photonic-crystal slow-light enhancement of nonlinear phase sensitivity” **Marin Soljacic**, S. Johnson, S.Fan, M.Ibanescu, E.Ippen, and J.D.Joannopoulos. ***Invited Paper in JOSA B*** Vol.19, 2052, (2002). Also appeared as an ***Invited Paper in Virtual Journal of Nanoscale Science & Technology*** 16th September 2002.

122. “Propagation of incoherent “white” light and modulation instability in noninstantaneous nonlinear media” Hrvoje Buljan, Antonio Siber, **Marin Soljacic**, and Mordechai Segev. ***Phys. Rev. E Rapid Comm.*** Vol.66, 035601(R), (2002).

123. “(1+1)-Dimensional modulation instability of spatially incoherent light” Detlef Kip, **Marin Soljacic**, Mordechai Segev, Suzanne Sears, and Demetrios N. Christodoulides. ***JOSA B*** Vol.19, 502, (2002).

124. “Pattern formation via symmetry breaking in nonlinear weakly correlated systems” Suzanne M. Sears, **Marin Soljacic**, Demetrios N. Christodoulides, and Mordechai Segev. ***Phys. Rev. E*** Vol.65, 036620, (2002).

125. “Integer and Fractional Angular Momentum Borne on Self-Trapped Necklace-ring Beams” **Marin Soljacic**, and Mordechai Segev. ***Phys. Rev. Lett.*** Vol.86, 420, (2001).

126. “Transverse instability of incoherent solitons in Kerr media” Juan P. Torres, Charalambos Anastassiou, Mordechai Segev, **Marin Soljacic**, and Demetrios N. Christodoulides. ***Phys. Rev. E Rapid Comm.*** Vol.65, 015601(R), (2001).

127. “Interactions between two-dimensional composite vector solitons carrying topological charges” Ziad H. Musslimani, **Marin Soljacic**, Mordechai Segev, and Demetrios N. Christodoulides ***Phys. Rev. E*** Vol.63, 066608, (2001).

128. “Delayed-Action Interaction and Spin-Orbit Coupling between Solitions” Ziad H. Musslimani, **Marin Soljacic**, Mordechai Segev, and Demetrios N. Christodoulides. ***Phys. Rev. Lett.*** Vol.86, No.5, 799, (2001).

129. “Low-loss asymptotically single-mode propagation in large-core OmniGuide fibers” Steven G. Johnson, Mihai Ibanescu, M. Skorobogatiy, Ori Weisberg, Torkel D. Engeness, **Marin Soljacic**, Steven A. Jacobs, J. D. Joannopoulos and Yoel Fink. ***Optics Express*** Vol.9, No.13, 748, (2001).

130. “Modulation Instability and Pattern Formation in Spatially Incoherent Light Beams” Detlef Kip, **Marin Soljacic**, Mordechai Segev, Evgenia Eugenieva, Demetrios N. Christodoulides. ***Science*** Vol.290, 495, (2000).

131. “Modulation instability of spatially-incoherent light beams and pattern formation in incoherent wave systems” Detlef Kip, **Marin Soljacic**, Mordechai Segev, Evgenia Eugenieva, Demetrios N. Christodoulides. ***Optics & Photonics News*** (***Optics in 2000***: special end-of-year issue), 34, December (2000).

132. "Eliminating the Transverse Instabilities of Kerr Solitons" Charalambos Anastassiou, **Marin Soljacic**, Mordechai Segev, Eugenia D. Eugenieva, Demetrios N. Christodoulides, Detlef Kip, Ziad H. Musslimani, and Juan P. Torres. ***Phys. Rev. Lett.*** Vol.85, No.23, 4888, (2000).

133. "Bright Spatial Solitons on a Partially Incoherent Background" Tamer H. Coskun, Demetrios N. Christodoulides, Young-Rae Kim and Zhigang Chen, **Marin Soljacic**, and Mordechai Segev. ***Phys. Rev. Lett.*** Vol.84, No.11, 2374, (2000).

134. "Modulation Instability of Incoherent Beams in Non-Instantaneous Nonlinear Media" **Marin Soljacic**, Mordechai Segev, Tamer Coskun, Demetrios N. Christodoulides, and Ashvin Vishwanath. ***Phys. Rev. Lett.*** Vol.84, No.3, 467, (2000).

135. "Minimal Potential with Very Many Minima" **Marin Soljacic**, and Frank Wilczek. ***Phys. Rev. Lett.*** Vol.84, No.11, 2285, (2000).

136. "Composite Multi-Hump Vector Solitons Carrying Topological Charge" Ziad Musslimani, Mordechai Segev, Demetrios Christodoulides, and **Marin Soljacic**. ***Phys. Rev. Lett.*** Vol.84, No.6, 1164, (2000).

137. "Self-trapping of Electromagnetic Beams in Vacuum Supported by QED Nonlinear Effects" **Marin Soljacic**, and Mordechai Segev. ***Phys. Rev. A.*** Vol.62, 043817, (2000).

138. "Self-Trapping of Necklace-Ring Beams in Self-Focusing Kerr Media" **Marin Soljacic**, and Mordechai Segev. ***Phys. Rev. E.*** Vol.62, 2810, (2000).

139. "Cantor Set Fractals From Solitons" Suzanne Sears, **Marin Soljacic**, Mordechai Segev, Dmitriy Krylov, and Keren Bergman. ***Phys. Rev. Lett.*** Vol.84, 1902, (2000).

140. "Self-Similarity and Fractals in Soliton-Supporting Systems" **Marin Soljacic**, Mordechai Segev, and Curtis R. Menyuk. ***Phys. Rev. E Rapid Comm.*** Vol.61, 1048, (2000).

141. "Self-Similarity and Fractals driven by Soliton Dynamics" **Marin Soljacic**, Suzanne Sears, Mordechai Segev, Dmitry Krylov, and Keren Bergman. ***Invited overview article in Photonics Science News***, Volume 5, Issue 1, (1999).

142. "Self-Trapping of Necklace Beams in Self-Focusing Kerr Media" **Marin Soljacic**, Suzanne Sears, and Mordechai Segev. ***Phys. Rev. Lett.*** Vol.81, No.22, 4851, (1998).

143. "Supernatural Inflation: Inflation from Supersymmetry with No (Very) Small Parameters" Lisa Randall, **Marin Soljacic**, and Alan H. Guth. ***Nuclear Physics*** B472, 377-408, (1996).

144. "Wireless energy transfer using repeater resonators"Aristeidis Karalis, Andre B. Kurs, Qiang Li, Katherine L. Hall, Morris P. Kesler, Eric Giler, **Marin Soljacic**, and Steven Ganem. U.S. patent number 8,587,155, issued in November 2013.

Patents (USA Only: Foreign Patents Not Included)

Search: http://www.mit.edu/~soljacic/patents.html on 2/9/2014 3:08 PM

1. "Wireless energy transfer systems"Aristeidis Karalis, Andre B. Kurs, Andrew J. Campanella, Konrad J. Kulikowski, Katherine L. Hall, Morris P. Kesler, **Marin Soljacic**, and Qiang Li. U.S. patent number 8,629,578, issued in January 2014.
2. "Wireless energy transfer systems"Aristeidis Karalis, Andre B. Kurs, Andrew J. Campanella, Konrad J. Kulikowski, Katherine L. Hall, Qiang Li, **Marin Soljacic**, and Morris P. Kesler. U.S. patent number 8,618,696, issued in December 2013.
3. "Wireless energy transfer using repeater resonators"Aristeidis Karalis, Andre B. Kurs, Qiang Li, Katherine L. Hall, Morris P. Kesler, Eric Giler, **Marin Soljacic**, and Steven Ganem. U.S. patent number 8,587,155, issued in November 2013.
4. "Wireless energy transfer using high Q resonators for lighting applications"Aristeidis Karalis, Andre B. Kurs, David Schatz, Katherine L. Hall, Morris P. Kesler, Eric Giler, **Marin Soljacic**, and Konrad J. Kulikowski. U.S. patent number 8,587,153, issued in November 2013.
5. "Wireless energy transfer using object positioning for improved k"Aristeidis Karalis, Andre B. Kurs, Andrew J. Campanella, Konrad J. Kulikowski, Katherine L. Hall, **Marin Soljacic**, and Morris P. Kesler. U.S. patent number 8,569,914, issued in October 2013.
6. "Integrated silicon-based nonlinear photodetector"Jorge Bravo Abad, Erich Ippen, and **Marin Soljacic**. U.S. patent number 8,515,216, issued in August 2013.
7. "Wireless energy transfer resonator kit"Aristeidis Karalis, Andre B. Kurs, Andrew J. Campanella, David Schatz, Konrad J. Kulikowski, Katherine L. Hall, Morris P. Kesler, Qiang Li, Ron Fiorello, **Marin Soljacic**, and Eric Giler. U.S. patent number 8,487,480, issued in July 2013.
8. "Wireless energy transfer using variable size resonators and system monitoring"Aristeidis Karalis, Andre B. Kurs, Andrew J. Campanella, Konrad J. Kulikowski, Morris P. Kesler, **Marin Soljacic**, and Katherine L. Hall. U.S. patent number 8,482,158, issued in July 2013.
9. "Wireless energy transfer with high-Q resonators using field shaping to improve K"Aristeidis Karalis, Andre B. Kurs, Andrew J. Campanella, Konrad J. Kulikowski, Morris P. Kesler, **Marin Soljacic**, and Katherine L. Hall. U.S. patent number 8,476,788, issued in July 2013.
10. "Surface-PlasmonoDielectric-polaritonic devices and systems"John D. Joannopoulos, Aristeidis Karalis, and **Marin Soljacic**. U.S. patent number 8,472,771, issued in June 2013.
11. "Wireless energy transfer over distance using field shaping to improve the coupling factor"Aristeidis Karalis, Andre B. Kurs, Andrew J. Campanella, Konrad J. Kulikowski, Morris P. Kesler, **Marin Soljacic**, and Katherine L. Hall. U.S. patent number 8,471,410, issued in June 2013.
12. "Tunable Wireless energy transfer for outdoor lighting applications"Aristeidis Karalis, Andre B. Kurs, Andrew J. Campanella, David Schatz, Katherine L. Hall, Eric Giler, **Marin Soljacic**, and Morris P. Kesler. U.S. patent number 8,466,583, issued in June 2013.
13. "Wireless energy transfer using conducting surfaces to shape field and improve K"Aristeidis Karalis, Andre B. Kurs, Andrew J. Campanella, Konrad J. Kulikowski, Katherine L. Hall, **Marin Soljacic**, and Morris P. Kesler. U.S. patent number 8,461,722, issued in June 2013.
14. "Wireless energy transfer using object positioning for low loss"Aristeidis Karalis, Andre B. Kurs, Andrew J. Campanella, Konrad J. Kulikowski, Katherine L. Hall, Morris P. Kesler, **Marin Soljacic**, and Morris P. Kesler. U.S. patent number 8,461,721, issued in June 2013.

15. “Wireless energy transfer using conducting surfaces to shape fields and reduce loss”Aristeidis Karalis, Andre B. Kurs, Andrew J. Campanella, Konrad J. Kulikowski, Katherine L. Hall, Morris P. Kesler, **Marin Soljacic**, and Morris P. Kesler. U.S. patent number 8,461,720, issued in June 2013.

16. “Wireless energy transfer systems”Aristeidis Karalis, Andre B. Kurs, Andrew J. Campanella, David Schatz, Konrad J. Kulikowski, Katherine L. Hall, Morris P. Kesler, Eric Giler, Frank Pergal, Ron Fiorello, Qiang Li, and **Marin Soljacic**. U.S. patent number 8,461,719, issued in June 2013.

17. “Multi-resonator Wireless energy transfer for exterior lighting”Aristeidis Karalis, Andre B. Kurs, Andrew J. Campanella, Konrad J. Kulikowski, Katherine L. Hall, Morris P. Kesler, Qiang Li, and **Marin Soljacic**. U.S. patent number 8,441,154, issued in May 2013.

18. “Low AC resistance conductor designs”Aristeidis Karalis, Andre B. Kurs, Andrew J. Campanella, David Schatz, Herbert T. Lou, Konrad J. Kulikowski, Katherine L. Hall, Morris P. Kesler, Ron Fiorello, **Marin Soljacic**, and Eric Giler. U.S. patent number 8,410,636, issued in April 2013.

19. “Wireless energy transfer across variable distances”John D. Joannopoulos, Aristeidis Karalis, and **Marin Soljacic**. U.S. patent number 8,400,024, issued in March 2013.

20. “Wireless energy transfer with high-Q capacitively loaded conducting loops”John D. Joannopoulos, Aristeidis Karalis, and **Marin Soljacic**. U.S. patent number 8,400,023, issued in March 2013.

21. “Wireless energy transfer with high-Q similar resonant frequency resonators”John D. Joannopoulos, Aristeidis Karalis, and **Marin Soljacic**. U.S. patent number 8,400,022, issued in March 2013.

22. “Wireless energy transfer with high-Q sub-wavelength resonators”John D. Joannopoulos, Aristeidis Karalis, and **Marin Soljacic**. U.S. patent number 8,400,021, issued in March 2013.

23. “Wireless energy transfer with high-Q devices at variable distances”John D. Joannopoulos, Aristeidis Karalis, and **Marin Soljacic**. U.S. patent number 8,400,020, issued in March 2013.

24. “Wireless energy transfer with high-Q from more than one source”John D. Joannopoulos, Aristeidis Karalis, and **Marin Soljacic**. U.S. patent number 8,400,019, issued in March 2013.

25. “Wireless energy transfer with high-Q at high efficiency”John D. Joannopoulos, Aristeidis Karalis, and **Marin Soljacic**. U.S. patent number 8,400,018, issued in March 2013.

26. “Wireless energy transfer for computer peripheral applications”Aristeidis Karalis, Andre B. Kurs, Andrew J. Campanella, Konrad J. Kulikowski, Katherine L. Hall, Morris P. Kesler, Qiang Li, and **Marin Soljacic**. U.S. patent number 8,400,017, issued in March 2013.

27. “Wireless energy transfer over a distance at high efficiency”John D. Joannopoulos, Aristeidis Karalis, and **Marin Soljacic**. U.S. patent number 8,395,283, issued in March 2013.

28. “Wireless non-radiative energy transfer”John D. Joannopoulos, Aristeidis Karalis, and **Marin Soljacic**. U.S. patent number 8,395,282, issued in March 2013.

29. “Optical devices having controlled nonlinearity”Peter Bermel, Alejandro W. Rodriguez, **Marin Soljacic**, and John D. Joannopoulos. U.S. patent number 8,369,670, issued in February 2013.

30. “Efficient near-field Wireless energy transfer using adiabatic system variations”Aristeidis Karalis, Rafif E. Hamam, John D. Joannopoulos, and **Marin Soljacic**. U.S. patent number 8,362,651, issued in January 2013.

31. “Wireless energy transfer using magnetic materials to shape field and reduce loss” Aristeidis Karalis, Andre B. Kurs, Andrew J. Campanella, Konrad J. Kulikowski, Katherine L. Hall, **Marin Soljacic**, and Morris P. Kesler. U.S. patent number 8,324,759, issued in December 2012.

32. “Wireless energy transfer using field shaping to reduce loss” Aristeidis Karalis, Andre B. Kurs, Andrew J. Campanella, Konrad J. Kulikowski, Katherine L. Hall, **Marin Soljacic**, and Morris P. Kesler. U.S. patent number 8,304,935, issued in November 2012.

33. “Efficient terahertz sources based on difference-frequency generation in triply-resonant photonic resonators” Jorge Bravo-Abad, Ian B. Burgess, John D. Joannopoulos, Steven G. Johnson, Marko Loncar, Murray W. McCutcheon, Alejandro W. Rodriguez, **Marin Soljacic**, and Yinan Zhang. U.S. patent number 8,285,091, issued in October 2012.

34. “Photonic crystal fibers having a preferred bending plane and systems that use such fibers” James Goell, **Marin Soljacic**, Steven A. Jacobs, Tairan Wang, Gokhan Ulu, Burak Temelkuran, and Steven G. Johnson. U.S. patent number 8,280,212, issued in October 2012.

35. “Optimized cascaded raman fiber-based laser source for high efficiency mid-infrared spectral generation” Peter T. Rakich, **Marin Soljacic**, and Yoel Fink. U.S. patent number 8,189,257, issued in May 2012.

36. “Wireless energy transfer” Aristeidis Karalis, Andre B. Kurs, Robert Moffatt, John D. Joannopoulos, Peter H. Fisher, and **Marin Soljacic**. U.S. patent number 8,097,983, issued in January 2012.

37. “Wireless non-radiative energy transfer” John D. Joannopoulos, Aristeidis Karalis, and **Marin Soljacic**. U.S. patent number 8,084,889, issued in December 2011.

38. “Wireless energy transfer including interference enhancement” Aristeidis Karalis, Rafif E. Hamam, John D. Joannopoulos, and **Marin Soljacic**. U.S. patent number 8,076,801, issued in December 2011.

39. “Wireless non-radiative energy transfer” John D. Joannopoulos, Aristeidis Karalis, and **Marin Soljacic**. U.S. patent number 8,076,800, issued in December 2011.

40. “Nonlinear harmonic generation and devices in doubly-resonant Kerr cavities” Hila Hashemi, Alejandro W. Rodriguez, **Marin Soljacic**, Steven G. Johnson, and John D. Joannopoulos. U.S. patent number 8,045,257, issued in October 2011.

41. “Wireless energy transfer using planar capacitively loaded conducting loop resonators” Andre B. Kurs, Aristeidis Karalis, Katherine L. Hall, Morris P. Kesler, and **Marin Soljacic**. U.S. patent number 8,035,255, issued in October 2011.

42. “Wireless non-radiative energy transfer” John D. Joannopoulos, Aristeidis Karalis, and **Marin Soljacic**. U.S. patent number 8,022,576, issued in September 2011.

43. “Wireless energy transfer” Aristeidis Karalis, Andre B. Kurs, Robert Moffatt, John D. Joannopoulos, Peter H. Fisher, and **Marin Soljacic**. U.S. patent number 7,825,543, issued in November 2010.

44. “Efficient harmonic generation and frequency conversion in multimode cavities” Alejandro Rodriguez, John D. Joannopoulos, Steven G. Johnson, and **Marin Soljacic**. U.S. patent number 7,768,694, issued in August 2010.

45. “Wireless non-radiative energy transfer” John D. Joannopoulos, Aristeidis Karalis, and **Marin Soljacic**. U.S. patent number 7,741,734, issued in June 2010.

46. “One-way waveguides using gyrotropic photonic crystals” Zheng Wang, Yidong Chong, John D. Joannopoulos, and **Marin Soljacic**. U.S. patent number 7,689,068, issued in March 2010.

47. “Efficient Terahertz sources by optical rectification in photonic crystals and metamaterials exploiting tailored transverse dispersion relations” Rafif Hamam, Mihai Ibanescu, Evan Reed, Peter Rakich, Steven G. Johnson, Erich P. Ippen, John D. Joannopoulos, and **Marin Soljacic**. U.S. patent number 7,421,171, issued in September 2008.

48. “Surface-Plasmon Index Guided (SPIG) Waveguides” **Marin Soljacic**, Elefterios Lidorikis, Kerwyn C.Huang, Evan Reed, David Chan, Yoel Fink, J.D. Joannopoulos, and Aristeidis Karalis. U.S. patent number 7,184,641, issued in February 2007.

49. "Coherent generation, Conversion, and Modulation of Electromagnetic Radiation Using Shock Waves or Solitons Propagating Through Polaritonic or Excitonic Materials" Evan J. Reed, **Marin Soljacic**, and J.D. Joannopoulos. U.S. patent number 7,116,458, issued in October 2006.

50. "Using Electro-Magnetically Induced Transparency in Photonic Crystal Cavities to Obtain Huge Non-linear Effects" **Marin Soljacic**, Lene Vestergaard Hau, and J.D. Joannopoulos. U.S. patent number 7,031,585, issued in April 2006.

51. "Gap-Soliton Devices in Photonic Crystal Fibers" Elefterios Lidorikis, **Marin Soljacic**, Mihai Ibanescu, Yoel Fink, and J.D. Joannopoulos. U.S. patent number 7,187,832, issued in March 2007.

52. "Using Optical Solitons to Increase Figure-Of-Merit of Laser Beam Deflection Devices" **Marin Soljacic**, Mordechai Segev, Chiyan Luo, and J.D. Joannopoulos. U.S. patent number 7,027,681, issued in April 2006.

53. "Shock wave modulation and control of electromagnetic radiation [shock-wave mechanism]" Evan J. Reed, **Marin Soljacic**, Steven G. Johnson, Maksim Skorobogatiy, and J.D.Joannopoulos. U.S. patent number 6,809,856, issued in October 2004.

54. "Shock wave modulation and control of electromagnetic radiation [nonlinear mechanism]" Evan J. Reed, **Marin Soljacic**, Steven G. Johnson, Maksim Skorobogatiy, and J.D.Joannopoulos. U.S. patent number 7,079,308, issued in July 2006.

55. "Photonic Crystal Waveguides Having Tailored Dispersion Profiles" Torkel Engeness, Steven Johnson, Mihai Ibanescu, Yoel Fink, Ori Weisberg, J.D.Joannopolous, Maksim Skorobogatiy, **Marin Soljacic**, and Steven A. Jacobs. U.S. patent number 6,895,154, issued in May 2005.

56. "Mach-Zehnder Interferometer Using Photonic Bandgap Crystals" **Marin Soljacic**, Shanhui Fan, Mihai Ibanescu, Steven G. Johnson, and J.D.Joannopoulos. U.S. patent number 6,917,431, issued in July 2005.

57. "High Index Contrast Fiber Waveguides and Applications [devices based on axial modulation and high-Q]" **Marin Soljacic**, Mihai Ibanescu, Torkel Engeness, Maksim Skorobogatiy, Steven Johnson, Ori Weisberg, Yoel Fink, Rokan U. Ahmad, Lori Pressman, Wesley A. King, Emilia Anderson, and J.D.Joannopoulos. U.S. patent number 6,898,359, issued in May 2005.

58. "High Index Contrast Fiber Waveguides and Applications [TIR devices]" Rokan U. Ahmad, **Marin Soljacic**, Mihai Ibanescu, Torkel Engeness, Maksim Skorobogatiy, Steven G. Johnson, Ori Weisberg, Yoel Fink, Lori Pressman, Wesley A. King, Emilia Anderson, and J.D.Joannopoulos. U.S. patent number 6,788,864, issued in September 2004.

59. "High Index Contrast Fiber Waveguides and Applications[codrawing rules]" Wesley King, Emilia Anderson, **Marin Soljacic**, Mihai Ibanescu, Torkel Engeness, Maksim Skorobogatiy, Steven G. Johnson, Ori Weisberg, Yoel Fink, Rokan U. Ahmad, and Lori Pressman. U.S. patent number 6,801,698, issued in October 2004.

60. "Low-loss photonic crystal waveguide having large core radius [continuation of U.S. patent 6,625,364]" Steven G. Johnson, Mihai Ibanescu, Ori Weisberg, Yoel Fink, J.D.Joannopolous, Maksim Skorobogatiy, Torkel Engeness, **Marin Soljacic**, and Steven A. Jacobs. U.S. patent number 7,072,553, issued in July 2006.

61. "Low-loss photonic crystal waveguide having large core radius" Steven G. Johnson, Mihai Ibanescu, Ori Weisberg, Yoel Fink, J.D.Joannopolous, Maksim Skorobogatiy, Torkel Engeness, **Marin Soljacic**, and Steven A. Jacobs. U.S. patent number 6,625,364, issued in September 2003.

"Wireless Energy Transfer using Repeater Resonators"

Aristeidis Karalis, Andre B. Kurs, Qiang Li, Katherine L. Hall, Morris P. Kesler, Eric Giler, Marin Soljacic, and Steven Ganem.

US Patent No: 8,587,155
Issued date: **Nov 19, 2013**
filing date: **Mar 10, 2010**
serial no: **12/720,866**
status: **In Force**

Described herein are improved configurations for a lighting system with wireless power transfer that includes a source high-Q magnetic resonator coupled to a power source and generating an oscillating magnetic field, at least one device high-Q magnetic resonator configured to convert said oscillating magnetic field to electrical energy used to power a light coupled to the at least one device resonator, and at least one repeater resonator, larger than the device resonator, wherein the repeater resonator is positioned further from the source resonator than the device resonator and improves the power transfer efficiency between the source resonator and the device resonator.

Claims

1. A lighting system with wireless power transfer comprising: a source high-Q magnetic resonator coupled to a power source and generating an oscillating magnetic field; at least one device high-Q magnetic resonator configured to convert said oscillating magnetic field to electrical energy used to power a light coupled to the at least one device resonator; and at least one repeater resonator; wherein the repeater resonator is positioned further from the source resonator than the device resonator and improves the power transfer efficiency between the source resonator and the device resonator.

2. The system of claim 1, wherein the source resonator is mounted on a wall below a cabinet and the device resonator is mounted above the source resonator under a cabinet.

3. The system of claim 2, wherein the repeater resonator is positioned inside a cabinet above the device resonators.

4. The system of claim 3, wherein the source resonator and the device resonators are separated by more than 10 cm.

5. The system of claim 3, wherein the source resonator and the device resonators are separated by more than 20 cm.

6. The system of claim 1, wherein the source resonator and the device resonators are tuned to a substantially same resonant frequency.

7. The system of claim 6, further comprising power and control circuitry coupled to the repeater resonator and configured to detune the resonator from said resonant frequency.

8. The system of claim 7, wherein said repeater resonator is detuned when voltage levels in the repeater resonator exceed a predetermined threshold.

9. The system of claim 1, wherein the source has a quality factor Q>100.

10. The system of claim 1, wherein the source resonator is integrated into a cover of an electrical outlet.

11. The system of claim 1, wherein the source resonator plugs into a household electrical outlet.

12. The system of claim 1, comprising multiple lighting fixtures.

13. The system of claim 1, comprising multiple repeater resonators.

14. The system of claim 1, wherein a characteristic size of the repeater resonator is larger than the smallest of the characteristic sizes of the at least one device resonator.

15. The system of claim 1, wherein the repeater resonator has a quality factor Q>100.

16. The system of claim 1, wherein the repeater resonator has a quality factor Q<100.

17. The system of claim 1, wherein a characteristic size of the source resonator is approximately 10 cm.

18. The system of claim 1, wherein a characteristic size of the source resonator is approximately 15 cm.

19. The system of claim 1, wherein the source resonator is a capacitively loaded loop resonator.

20. The system of claim 1, where a characteristic size of the repeater resonator is approximately 20 cm.

21. The system of claim 1, where a characteristic size of the repeater resonator is approximately 40 cm.

22. The system of claim 1, where the repeater resonator is sized to fit inside a cabinet.

23. The system of claim 1, where the repeater resonator is sized to fit inside a drawer.

24. The system of claim 1, where the repeater resonator is a capacitively loaded loop resonator.

25. A wireless power transfer system comprising: a high-Q source resonator coupled to a power source and generating an oscillating magnetic field, a high-Q device resonator coupled to an energy drain and configured to convert said oscillating magnetic field to electrical energy, and a repeater resonator; wherein the repeater resonator is positioned to improve the coupling between the source resonator and the device resonator and the repeater resonator is positioned further away from the source resonator than the device resonator.

26. The wireless power transfer system of claim 25, wherein the repeater resonator is larger than the device resonator.

27. The system of claim 25, wherein the repeater resonator has a quality factor $Q>100$.

28. The system of claim 25, wherein the repeater resonator has a quality factor $Q<100$.

29. The system of claim 25, wherein the resonators of the system have a quality factor $Q>100$.

"Wireless energy transfer systems"

Aristeidis Karalis, Andre B. Kurs, Andrew J. Campanella, Konrad J. Kulikowski, Katherine L. Hall, Qiang Li, Marin Soljacic, and Morris P. Kesler.

US Patent No: 8,618,696
Issued date: **Dec 31, 2013**
filing date: **Feb 21, 2013**
serial no **13/773,022**
Status: **In Force**

A wireless power supply includes a source magnetic resonator, connected to a power source and configured to exchange power wirelessly via a wireless power transfer signal with at least one device magnetic resonator integrated into at least one peripheral component of a computer and a processor configured to adjust the operating point of the wireless power supply wherein power is transferred non-radiatively from the wireless power supply to the at least one device magnetic resonator and wherein the power supply forms a part of the computer.

Claims

1. A wireless power supply comprising; a source magnetic resonator, connected to a power source and configured to exchange power wirelessly via a wireless power transfer signal with at least one device magnetic resonator integrated into at least one peripheral component of a computer; anda processor configured to adjust the operating point of the wireless power supply wherein power is transferred non-radiatively from the wireless power supply to the at least one device magnetic resonator and wherein the power supply forms a part of the computer.

2. The wireless power supply of claim 1, wherein the processor is configured to adjust a frequency of the wireless power transfer signal.

3. The wireless power supply of claim 1, wherein the processor is configured to adjust an impedance matching network of the wireless power supply.

4. The wireless power supply of claim 1, wherein the processor is configured to adjust a power converter of the wireless power supply.

5. The wireless power supply of claim 1, wherein the processor is configured to adjust an amplifier of the wireless power supply.

6. The wireless power supply of claim 1, wherein the wireless power supply is configured to send wireless control signals to the at least one device resonator.

7. The wireless power supply of claim 1, wherein the wireless power supply is configured to receive wireless signals from the at least one device resonator.

8. The wireless power supply of claim 1, wherein integrated device magnetic resonator comprises power and control circuitry.

9. The wireless power supply of claim 8, wherein the device magnetic resonator power and control circuitry comprises a processor.

10. The wireless power supply of claim 9, wherein the device magnetic resonator processor is configured to provide information to the wireless power supply.

11. The wireless power supply of claim 1, wherein the at least one computer peripheral comprises at least one energy storage unit.

12. The wireless power supply of claim 11, wherein the at least one energy storage unit is a battery.

13. The wireless power supply of claim 11, wherein the at least one energy storage unit is a supercapacitor.

14. The wireless power supply of claim 1, wherein the processor controls a battery charging circuit of at least one computer peripheral.

15. The wireless power supply of claim 1, further comprising at least one repeater resonator.

16. The wireless power supply of claim 15, wherein the at least one device magnetic resonator is configured to operate as a wireless source to at least one other magnetic resonator in an active area.

17. A wirelessly powered computer peripheral, comprising: a tunable device magnetic resonator; a means for transferring information wirelessly; and a means for adjusting a parameter of the tunable device resonator; wherein the peripheral is selected from the group consisting of a keyboard, a mouse and a touch surface.

18. A wirelessly powered computer peripheral, comprising: a magnetic resonator, wherein the magnetic resonator is configurable to operate as a device resonator for capturing energy and delivering power to the computer peripheral in a first active area and as a repeater resonator for improving energy transfer to another computer peripheral in a second active area.

19. The computer peripheral from claim 18, wherein the magnetic resonator is configurable to operate simultaneously as a device resonator and a repeater resonator.

20. The computer peripheral from claim 18, wherein the magnetic resonator is periodically switched from a repeater function to an energy capture function.

21. The computer peripheral from claim 18, wherein the peripheral comprises a keyboard.

22. The computer peripheral from claim 18, wherein the peripheral comprises a monitor.

23. The computer peripheral from claim 18, wherein the peripheral comprises a touch screen.

"Wireless energy transfer systems"

Aristeidis Karalis, Andre B. Kurs, Andrew J. Campanella, Konrad J. Kulikowski, Katherine L. Hall, Morris P. Kesler, Marin Soljacic, and Qiang Li.

US Patent No: 8,629,578
Issued date: **Jan 14, 2014**
filing date: **Feb 21, 2013**
serial no: **13/773,011**
status: **In Force**

A wireless power transfer system for computer peripherals, includes a source magnetic resonator, integrated into a source station and connected to a power source and power and control circuitry, and a device magnetic resonator, integrated into a computer peripheral wherein power is transferred non-radiatively from the source magnetic resonator to the device magnetic resonator, and wherein the source magnetic resonator is configured to transfer power during predefined intervals.

Claims:

1. A wireless power transfer system for computer peripherals, comprising:a source magnetic resonator, integrated into a source station and connected to a power source and power and control circuitry, anda device magnetic resonator, integrated into a computer peripheral;wherein power is transferred non-radiatively from the source magnetic resonator to the device magnetic resonator, and wherein the source magnetic resonator is configured to transfer power during predefined intervals.

2. The system of claim 1, wherein the source magnetic resonator is configured to transfer power when the computer peripheral is not in use.

3. The system of claim 1, wherein the source magnetic resonator is configured to transfer power during predefined times of a day.

4. The system of claim 1, wherein the source magnetic resonator is configured to transfer power when the computer peripheral is within an energy transfer range of the source.

5. The system of claim 1, wherein the device magnetic resonator comprises shielding to reduce a magnetic field interaction with lossy materials forming a part of the peripheral.

6. The system of claim 1, wherein the computer peripheral is powered directly by the energy transferred to the device magnetic resonator.

7. A wirelessly powered computer peripheral, comprising: a device magnetic resonator, and a repeater resonator, wherein the repeater resonator is positioned to enable the coupling to a second computer peripheral.

8. The peripheral of claim 7, wherein the peripheral is a computer keyboard.

9. The peripheral of claim 8, wherein the second peripheral is a computer mouse and the repeater resonator is positioned to enable the transfer of power to the computer mouse positioned on one side of the keyboard.

10. The peripheral of claim 7, wherein the peripheral is a touch surface.

11. The peripheral of claim 7, wherein the computer peripheral includes at least one rechargeable battery that is charged by a wireless power transfer system.

12. The peripheral of claim 7, wherein the computer peripheral includes at least one energy storage unit that is energized by a wireless power transfer system.

13. The peripheral of claim 7, wherein the computer peripheral includes at least one super capacitor that is charged by a wireless power transfer system.

14. A wirelessly powered computer peripheral, comprising: a magnetic resonator, wherein the magnetic resonator is configurable to operate as a device resonator for capturing energy and delivering power to the computer peripheral and as a repeater resonator for enabling energy transfer to another computer peripheral from an external wireless energy source.

15. The computer peripheral of claim 14, wherein the magnetic resonator is configurable to operate simultaneously as a device resonator and a repeater resonator.

16. The computer peripheral of claim 14, wherein the magnetic resonator may be periodically switched from a repeater function to an energy capture function.

17. The computer peripheral of claim 14, wherein the peripheral is a computer keyboard.

18. The computer peripheral of claim 14, wherein the peripheral is a monitor.

19. The computer peripheral of claim 14, wherein the peripheral comprises a touch surface.

20. The computer peripheral of claim 14, wherein the peripheral comprises a light.

21. The computer peripheral of claim 14, wherein the peripheral is at least one of a smart phone, a tablet computer, a game console and a controller.

22. The computer peripheral of claim 14, wherein the peripheral is powered directly by the energy transferred to the device magnetic resonator.

23. The computer peripheral of claim 14, wherein the peripheral comprises at least one rechargeable battery that is charged by a wireless energy source.

24. The computer peripheral of claim 14, wherein the peripheral comprises at least one energy storage unit that is energized by the wireless energy source.

25. The computer peripheral of claim 14, wherein the peripheral includes at least one super capacitor that is charged by the wireless energy source.

Collectiveness of Visual and Z-infrared Spectrum in the Security Printing

Vilko Žiljak, Croatia

HATZ, Croatian Academy of Engineering

Review

INFRAREDESIGN® is an extension of theory and practice for printing inks, paper, canvas, leather, ceramics and various materials management, with a desire to "see" the reproductive print in the same way as the nature. Infra-red color management covers ultraviolet, visual and infrared part of the spectrum. This new approach allocates a new way of studying of the camouflage that respects the values of near infrared flora, fauna and minerals. Establishing twin dyes is the beginning of creating invisible images, dual information and community of broadened spectrum in the design of visual art domain. We are introducing and practising multicolor infrared method in security printing graphics, in the area of packaging and painting. Z value is proposed as the measure of absorption of light in the NIR Infra – Color Management.

Key words:

INFRAREDESIGN, VZ color space, ZRGB camera, Z value, Newspaper IR-print, CMYKIR separation.

1. INFRAREDESIGN®

The phenomenon, discovery, scientific excitement was created in 2008 with Ivana Žiljak dissertation which is a major driving force in the creation of new visual art and technology called INFRAREDESIGN®. Co-authored theory is today signed by Žiljak Jana Vujic, Klaudio Pap and I Vilko Žiljak. On behalf of the aforementioned colleagues, I set report about our work and results of a new way of separation, and unity of the visual (V) and infrared parts of spectrum (NIR).

Results of enormous experimental work have been published in books [1, 2] and publications [3 to 32], and at conferences. The first larger publication was published in the "Annual of the Croatian Academy of Engineering in 2008 [3]. On prototypes development, that is the basics of the patent, we engaged a dozen engineers from different organizations and specialities, especially from companies Fotosoft and FS. Testing and evidence of a number assertions about the new Infra-red color management are made in plants and on machines that treat a variety of mate-

* Corresponding author: vilko@ziljak.hr

rials, such as leather, cotton, thread, paper, ceramics and canvas for artistic painting. Worldwide recognition implies: awards in scientific discovery as well as for professional use as innovations in security printing, a new way of painting, a different approach to the field of camouflage in the defence industry, with the publication of a new Z variable in the journal "Infrared Physics & Technology" [4]. It has been reported several patents which are maintained under the numbers: EP2165844, HR: P20080466A, P20100201A, P20100201A, P 20100451A.

1. 1. V and Z Spectrum

INFRAREDDESIGN ® sets a discussion trough the associated terminology. V (Visual – RGB, 400-700 nm) spectrum provides a "tone color", immaterial color that we see experience or perceive. People perceive V range as a variety of tone colors. In opposite, the term "dye" is a substance which absorbs some light and reflects the rest. Dye reflection experiences its own (color) tone, intensity and saturation. When a matter is illuminated with a wavelength of 1000 nm, whose absorption is measured and expressed by the specfic value, designated as "Z parameter" [4]. Our eyes do not see the reflected light of 1000 nm, but this value was recorded with adjusted camera, Z camera. Such NIR image (near infrared spectrum image), can be seen as a gray tone, meaning reflected Z states of matter. Z camera allows us to experience the Z state of material, and we are talking to "see material" – illuminated by infrared wavelengths. We consider dyes that are applied to the canvas, ceramics, leather and paper, silk. For these materials we measure reflectance of V light and in addition reflection in the Z point. Z spectrum is interpreted as a grey scale [4]. To the material first is embedded a description of the visual RGB values, followed by the characteristic of absorption and reflection in NIR wavelength.

Our work is focused on active managing of the coloured matter, called "dyes" in order to achieve a given or default absorption of light in the V and Z spectrum range. Object is observed with a separate V and Z sensitive cameras [5]. Each camera simultaneous records the image that is intended for it. Z camera "sees" by day and by night. Each camera records the image that is intended for it. Z camera "sees" by day and by night. It has an infrared illumination source, or uses NIR component of sunlight. Z camera misses the RGB spectrum. V camera registers only the RGB spectrum. "Twin colors and dyes" is introduced as the new term." Twins are two or more dyes that have the same visual experience, but different Z values.

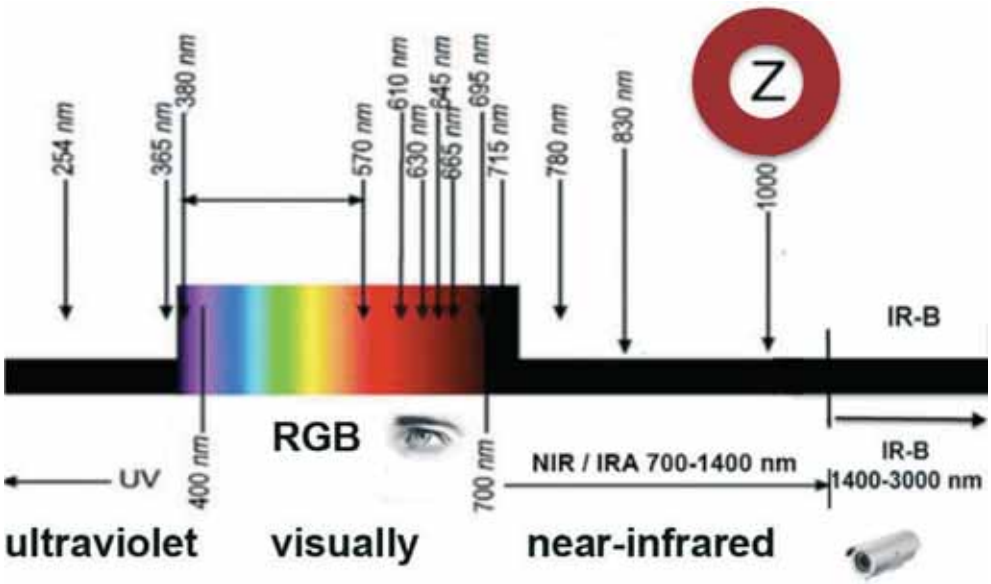


Fig. 1 – RGB and NIR – a barrier points for Projektina

Some devices for the analysis of ultraviolet, visual and near infrared spectrum allow selective filtering [6]. Such scanning with the associated spectroscopy significantly improves the quality score after iterative mixing dyes with the aim of making twins dyes. Adding and subtracting spot dyes from the base of the "original sets" is big task in the future. Panton basic scales dyes, for example, have yet to be studied in INFRA-REDESIGN application. There are neither procedures for offset printing, nor for liquid dyes of flexo printing. We used spectrographic scanner Projektina [6] and its barrier points (Figure 1), that are starting points for planning and routing dyes composition in several states of twins. We have developed ourselves ZRGB cameras [5] that provided us a very precise determination of the NIR absorption.

1.2. IRD in the field of security prints

On the banknotes printing inks (dyes) with controlled properties, planned light absorption in the near-infrared light (NIR) are used. Coloured graphics are not applied in the NIR, except if only one selected color, which is derived from the "infrared dye." Such a ink is mixed as NIR spot dye, with more or less success in achieving similar tone, but just as the adjacent graphic. Not to make any mistakes in achieving the same tone, deliberately NIR dye is mixed with a different tone than other graphics on a banknote. Even if it is the same graphical form, as banknotes in denominations, design and graphics has become colorful. This method in NIR printing is called "blocking infrared printing". Fully NIR color balancing with

neighbouring dyes is difficult to sustain in the press. Perhaps this is the reason that there is no practicing of "hiding, double information, two prints in the same place." That was the situation until the practice of INFRAREDESIGN. And that's the fact that makes the uniqueness of our findings: On the print there is designed information of infinite number of tones and hues, especially with V characteristics, separately with special properties of the Z: Any graphic design, hidden from our eyes, hide from infrared surveillance cameras. And the graphics still exist, they can be identified in a planned and defined viewing conditions, ie recognition. Usually authors use the term "invisible reality."

Fig. 2 a,b,c – Multicolored graphics in barijerma: V (400-700nm), 715 nm, 1000 nm

The scanned prints at 1000 nm clearly show a second, hidden text. Figure 2 presents the state of the 715 nm with the included Z illumination (in the middle). At these wavelengths cyan dye responses still. We can see a mixture of blue / black tones. Since some spot dyes have a range of light absorption up to 750 nm, we introduced the Z value at 1000 nm, to make sure that we are working with two groups of dyes, V and Z of dyes. The first, V group consists of dyes which have no absorption properties above 750 nm. For various painting applications, on a variety of materials, this first group of dyes are coming with names of cyan, magenta and yellow. Although for these dyes is set the initial theory of colors and dyes, however, many variations are present. Such examples are dyes that come in the area of art paintings.

2. THEORY INFRAREDESIGN®

Six years ago, the first book INFRAREDESIGN was promoted ®, at the National University Library in Zagreb [1), authors: Ivana Žiljak, Klaudo Pap and Jana Žiljak Vujic. About the book the promoters were: Andrew Tribute, Visiting Professor at University of Arts London, Frank Romano come from America, Professor emeritus, School of Print Media, Rochester Institute of technology, USA; Anastasios E. Politis, Professor at Graphic Art and Media, Athens; Kurt Wolf, Detsche Drucker, Germany. In 2009, the second edition of the book INFRARED SECURITY GRAPHICS [2], the review was expanded with professors: prof.dr.sc. Darko Agić, Faculty of Graphic Arts, Zagreb, Prof.dr.sc. Aleš Hladnik, Faculty of Natural Sciences and Engineering, University of

Ljubljana, Prof.dr.sc. Vesna Kropar Vančina, Faculty of Graphic Arts, Zagreb. Books are offered for discussion about different management printing dyes that had been previously opposed the theory of subtractive and additive theory of colors and dyes.

IRD theory is based on the fact that the same visual color characteristics can be achieved by different methods of mixing colors. We mixed and blended the dyes that do not absorb light in the Z point. Such set of dyes we call V dyes. The second set of dyes absorbed both spectra: V and Z spectrum parts. First settings of IRD theory dealt with different amounts of carbon black component in exchange for CMY dyes [7]. There are developed a numerous mathematical models, called Infra-colorsetings. Specific area (domain) is determined for dyes for each combination of real dyes and materials where dyes are applied: especially for ceramic dyes, specifically for the leather treatment, especially for cotton and ink-jet printing. Dyes domain is determined by the size Z that can be achieved in a given connection of dyes and materials. Some mathematical models have been created to perform the maximum size of Z value, for all color tones [7, 8]. Mathematical models of spot dyes, which are derived from the process dyes, describe the polynomial dependence CMY on K for each tone separately [3, 8, 9, 11]. If printing techniques is with pure spot dyes, then such spot dyes must be prepared before printing, but with recipies obtained from the "twin colors and dyes." system

Conference "Printing & Design" was held in early February 2013th in Tuheljske toplice with the most comprehensive display of new technologies IRD ®. A wide series of theoretical discussion of the general theory of IRD ®, can be found in the works of experts from different institutions [12, 13]. Some works have been announced for doctoral theses since are opened many unresolved problems of management dyes in the visual spectrum with a controlled absorption of infrared light. The work of Igor Čajkušić from Art Academy in Split [14] is an attempt to explain the appearance of infrared light absorption by painting pigments. In this way it is creating the conditions for a "passive" and "active" IR painting that comes under the name INFRAREDART. Presented are the latest in art and design practice with double figures or images. Rich section of IRA exhibitions from Ivana Žiljak Stanimirović in galleries is presented at 2012th conference "Blaž Baromić" u Senju [15, 16, 17, 18].

3. Colours and Dyes

It has already been emphasized the difference between the colors and dyes. In practical printing, art work, there are offered in a variety of shades. Some groups are reaching recipes for thousands of color tones. Panton, TOYO91 for example, are offering their twenty basic dyes. They give instructions how to reach other dyes.

For now, none of these major dyes manufacturers do not offer recipes for making dyes twins regarded to Z value. We are engaged in setting up the theoretical and practical work, called INFRAREDESIGN® in order to develop a new way of managing colors and dyes for the visual and infrared spectrum. All this leads to the following topics: hiding images, information hiding, camouflage, anti-terrorist equipment, safety graphics: On a new IRD way.

3.1. The infrared properties of dyes in art painting

IRD theory is based on the press with at least two dyes to the same color perception, but different values of Z parameter. In this minimal requirement is desirable that two dyes have a high degree of contrast characteristics, which determines the absorption of infrared light. The general IRD theory it is desirable that there are two sets, two groups of dyes. Let us illustrate this in the application of INFRAREDART painting. The painter has many dyes, various color tones, visually. On one side are dyes that do not absorb infrared light, and on the other side is painters color palette with positive values of the Z parameter. The painter mixes dyes so that he achieves the same color tones (output) on both pallets. The first pallet is e.g. brown (dye) which does not absorb IR. On the other pallet is the same brown dye, but it absorbs in IR. Similarly, for variety of other tones, painter prepares dyes to obtain a wide range of colors. These sentences are supplemented by explanation of the differences between the terms of colors and dye. The first is what we experience, what we see. Dyes are materials that painter applies to the canvas. However, in the store (Figure 3, and Table 1) there is no dye whose Z value is zero. The real art is to create many tones, many dyes that would be placed in the first group. Such dyes would be ideal components for mixing and creating other tones. And in artistic painting, there it is a rich range of color tones.

Fig. 3 a,,b – Tempera in the visual and near-infrared light

Fig. 4 a,b – Acrylic painting "in the way InfraArt painting. V and Z state

The painter does not know mathematics, and mathematical models of mixing dyes [19]. This principle develops a new craft in creating pairs of twins that only artists develop with his painting talent and the need for expression but in their own way.

Artist achieved two stages of the picture. Merrily masking, almost naive. In masquerade background is a dream, intima, erotica hidden from our eyes; zRGB camera detects the 'masquerade idea', where everything is allowed.

3.2. CMYKIR separation

Process dyes cyan, magenta, yellow and carbon black form the basics of four-color printing [7, 20]. Carbon black dye, K, has the characteristic to absorb in NIR. The value of Z parameter is equal to one (100%). In opposite, black tones can be achieved with equal shares of C, M, and Y dyes. Such black dye does not absorb NIR. The value of the Z parameter is equal to zero, when the dye is so prepared. We operate with two black dyes with extreme Z sizes. If the black dye mixed with white dye, we get grey tone color or grey dye. System of process dyes have the property that certain portion of a mixture of C, M, Y can be replaced with a black dye. Equal values of C, M, Y can be replaced with a black dye. This replacement is possible for all shades of color that are derived from the C, M, Y, until one of the C, M, Y falls to zero. This is a domain, or IRD space with process dyes. All printing techniques rely on the method of replacing the chromatic colors C, M, Y with black. Since we have two black dyes, we can make two dyes with the same color tone but with two different Z values. There starts the idea of hiding the pictures from our eyes: The same color tones and responding differently in V and Z spectrum [21].

Most IRD printing technologies are using properties of replacing C, M, Y with black dyes to achieve the double condition, two images at the same place. IRD designer programmes amounts of C, M, Y, K dyes according to the participation of some black dyes. Some digital printers do not allow distinguishing black only with C, M, Y components from black with carbon black component. IRD system is not possible for such devices. In all other printing techniques where dyes are mixed outside the printing press, before printing, it is possible to achieve IRD effect. CMYKIR separation implies merging two images. Visual and infrared image. The first has CMYKIR separation only with CMY components. The second image is given as a grey scale. CMYKIR separations are using GCR method and its theory, but with a different plan of replacing CMY with K. To each element of the CMY image is determined separately calculated insertion of K colors. Quantity of carbon black dye is determined in the second, Z image. This process and the associated mathematical models implies that CMY dyes do not respond into the Z spectrum. Secondly, carbon black achieves an intense response in the Z range.

3.3. Properties of "ultraviolet dyes" in the infrared spectrum

IRD technology, in addition, has spread to dyes which are designated as "ultraviolet dyes." Although UV and IR are on opposite sides of the visual spectrum (Figure 1), these two areas form fellowship in highly valuable security printing. A special feature of UV dyes is changing color, depending on whether wavelengths the dye is highlighted. The same dye can be black in the visual spectrum and light green when is it illuminated with ultraviolet light, for example. With such dyes are marked numbers – numbering in Croatian Kuna banknotes. We see the two colors of the same colors. In our experiments (fig. 5), dark dye has absorption properties of infrared light. When observed with Z camera, print is grey. Depending on the amount of dyes applied, the print is darker or lighter. The effect is the opposite in the UV and IR light. What is the achievement of greater value Z, the image under UV light is brighter. There is an increasing coat of fluorescent dyes. For the of graphic safety experiments, there is new, vast range of dyes which are managed in three separate parts of spectrum, observed in three separate ways: UV, V and Z are different graphics [9, 21, 22].

If we want to achieve the hiding effect of two or more images with UV colors that have a positive response in the NIR spectrum, then we come into the space calculations of "UV, V and Z twins." The aim is to make such mixtures of dyes that have the same status in the visual spectrum: The same color. Triple state absorption and reflection in three situations Is programmed. It is managed with ink layer on the way to achieve invisible graphics planning to UV light, visual light or IR light. We emphasize the concept of "hiding". Only at this point there is the new value of URD theory and application for new security graphic [21]. UV and IR properties of

Fig. 5 a,b,c – Integration of the picturesque vineyard and Ozalj castle, scanned in the UV, V and Z spectrum

the dyes are great unexplored areas for which we have made prototype solutions with the unique aim: we have shown that it is possible to perform camouflage in three light parts of spectrum.

3.4. Twin dyes in the infrared, visual and ultraviolet spectrum

Infra colorseting is based on the development of twin colors and dyes. For the desired color tone there are two dyes produced. The color is demonstrated that consists of process C, M, Y and F (fluorescent) dyes from the group of "ultraviolet" dyes. F dye appears dark brown in the visual spectrum. It has the property of responding to yellow / orange status if illuminated with UV light. That dye also strongly absorbs near-infrared light. Therefore, it can be used in designing of dual information with IRD methods. Since this is F dye has a positive translation of V in the UV range, it is possible to design an additional third picture in the same place, as It was shown in section 3.3. A basic of preparation of UV / V / Z protective design is the creation of several dyes, dyes that have the same visual characteristics.

In Figure 5, there are 32 different prints, where each color is made of two dyes with the same color tone in the V spectrum. Round graphics are made up of C, M, Y

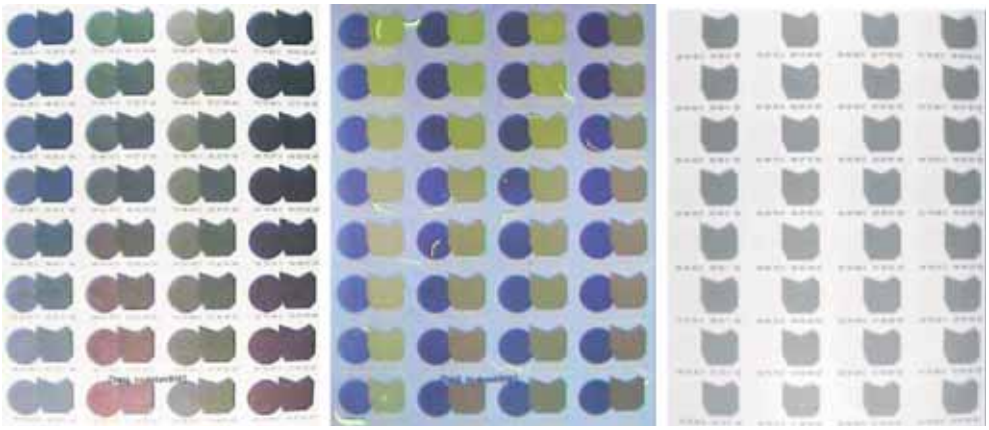


Fig. 6 a,b,c – Prints scanned in visual, ultraviolet and infrared spectrum

process colors. Octagonal graphics are composed of C, M, Y and F dyes. The first image is scanned in the visual condition mode. Pairs of dyes are made: Round graphic has almost the same spectrogram as an octagonal graphic in the visual spectrum.

The second image is scanned under ultraviolet light condition, at 360 nm. All 32 octagons have an equal share F dye: yellow / brown tone in VU spectrum. All of these graphics have participation of 40% of fluorescent dyes. Circular forms are shown as blue tone, regardless of the share C, M, Y components. The second image is visible, but the twins' pair's dyes appear different for our eyes. It is the characteristic of the chosen UV dye.

The third group, from fig. 5. shows the state after scanning at 1000 nm, which is called Z state. Since the C, M, Y dyes do not absorb in near-infrared light, there is no response from round graphics. Visible are only octagonal graphics that contain 40% F dyes. F dye, although declared as UV dye, has a strong absorption in the Z spectrum, what will be used for IRD hiding, the IRD information, and IRD double image.

The twin pairs with circular prints and dyes with octagonal graphics respond differently in the two spectra: UV and Z. This is a new approach to the study and application of IRD in security printing. These twins are different in V and Z part, but must be equal to V spectrum. Achieving twins' pairs is an iterative process of offset printing techniques, with the aim of determining the same color tone in the V spectrum, and for different share of C, M, Y, F components. Identification of the visual spectrum is performed by spectroscopic analysis, determining colorimetric values on principles for conventional GCR printing method. In these experiments, the participation of F dyes is equal for all colors. It was decided to set a yield of 40%, as

it is sufficient for distinct identification of Z graphics with ZRGB cameras. F dyes higher participation would reduce the range of different dyes, especially in brighter areas of the initial V image [22].

Experiments were conducted for other UV dark color. Especially for UV dye that starts from dark, almost black tone, until the yellow tone at 360 nm. There are set new procedures and methods of hiding and mutation the information, depending on the area of blocking and filtering the wavelength in the range of 200 to 1200 nm. Security graphics can be designed as multiple images at the same place, where they are mutually protecting from forgery.

4. Z value of the Materials

For conventional printing we developed procedures for inks and materials where the dyes will be applied. An expert, dedicated collection of properties was developed, called "colorsetting". They are embedded with software for processing and separation of images in the rendering process from RGB status in CMYKIR state, and are prepared for production of printing plates. It's a huge task, since for each combination of color / material/substrate a special colorsetting must be developed with dedicated degrees of GCR separation.

Till today, no one has developed neither delivered colorsetting that would include the absorption characteristic of the visual and infrared spectrum. Only in our works [4, 22, 23, 11] there are offered several mathematical models for plotters, digital printing, offset and flexo systems. These relations have a background in scientific papers, making IRD camouflage clothing, drugs protection packaging for pharmacist industry, the production of documents and print labels on polypropylene. We developed infra-colorseting that includes ultraviolet, visual and infrared. On this enormous task, we engaged experts with the aim of creating a knowledge base, and a base of mathematical models for INFRARED printing applications. We must point out that is no single, universal solution. We set out procedures for experiments with different dyes and materials, with the aim of finding the shortest path to the twin dyes combinations.

For the same printing technique, a plotter for example, for each material a completely different INFRA-colorsetting must be performed. Project IRD is very sensitive. When merging two images, the first test is visual. The secondary, Z image, must not be perceived. When you achieve this state of IRD task, we can say that the experimental phase of the work is on the right track. The second test is spectrometric. Since it is not allowed to see another image, Z image, it is satisfactorily to

analyse the situation of twins' visually. IR spectrogram is not very important, because it depends on the state of the camera that observes Z image, and amount of NIR light sources. The same situation is when working with UV dyes that respond to IR light. Visual (400 – 700 nm) spectrogram shows the direction necessary for corrections of participation C, M, Y dyes that are running in IRD application. This iterative experimental work is monitored and supported with great documentation, because dyes are changing under the influence of external light and moisture. After a period of time an eventual "outbreak" of Z image can be determined. It requires comparison of old and new spectra, to determine the experimental plan and to create a new Infra – colorsetting.

4.1. EXTENDED INFRA – Color Management in newspaper printing

Extended color management allows the visualization of quality prints in visual spectrum, without electronic checks. IRD ® is a combination of two images that may not be seen simultaneously, and that property can be used as a impression checkout quality in the task of identifying planned and executed dyes. Large newspaper rotations [24, 25] are carried out quality corrections during the printing process, without stopping printing. Since IRD ® integrates two graphics that are mutually hid; we exploit this property in the control register and coating of each individual process components. At the moment when one or another image becomes recognizable, it indicates that amount of dyes must be re-tuned up and repaired. The situation of "hidden graphics visibility" is easier than instrumental measuring of good coloring amount.

Tests have been carried out on the offset rotation by Printers Vjesnik in Zagreb. By offset process there are different color settings by rotation printing and those by the sheet printing. This can improve procedures "pegs" on the visual level control of volumes of dyes. Newspaper printing is the first evidence that there is no "post-print" treatment. No post treatment is needed for infrared effect. High circulation volumes do not allow experimentation during printing. First test Infra – colorseting were running on the newspaper printing edges, as Infra – wedges. Once we have established relationships twins' dyes for newsprint and accompanied dyes, we designed the safety information that is hidden from our eyes. First Infra – colorsetting is running on the edges of the newspaper. Prints were made in the 2009 in Zagreb. To the picture: Physicist in Cern (Switzerland), we added Infra image – Einstein. An extensive work with more applications in 2011 is published In India, [25].

A plan for appearance difference of the same tones can be programmed prior to printing system as "twins". The twins differ in their parameter of Z values. Extreme cases X0 and Xmax [17] were the beginning of development of the dedicated algorithm for CMYKIR separation [8].

Fig. 7 a,b – Newspaper print (27.8.2010.) with dual graphics, design "physicists and Einstein" at CERN.

It is proposed to design graphics that are mutually hidden, so that both V and Z graphics are clearly visible on each printing form or offset plates, in their characteristic appearance. The same should appear on the graphic film. In final impression Z picture is not visible in visible spectrum. Re-recognition of two graphics, although there is no need for it, is derived from ZRGB camera, that can be integral part of the newspaper plant rotation. This is a new IRD® result, which is based on the proposal to expand the color space from 400 to 1000 nm [29]. Successful tests in a news rotation are showing that the IRD® is applicable also when experiments in conventional manner are not permitted, because it could cause higher production costs. Dyes are spectroscopic studied before the start of the new IRD® applications. Twins dyes are mixed precisely in order to support the dedicated IRD® colorsetting for the target printing technology.

4.2. Flexographic printing on polypropylene

VZ IRD® printing technology was tested on transparent materials in flexo [22] with relation to liquid dyes. Such printing technology is quite different from offset printing, screen printing and digital printing. Mathematical models imply linear regression equations, adjusted, after iterative tests that require time consumption prepress, and many costly production of flexographic printing forms. In the middle of packaged cookies, added is the IR protective rosette, in many colors. The strangeness is that this part of the print is not shimmed by coverage with a white dye. This did not achieve the planned color, but has performed transparency. Packed cookies can be seen in the spaces between thin colored lines.

Detail of the rosette shows its linear structure. Value of the positive Z condition occurs only where the dyes are. Therefore, text in the infrared graphics is interrupted. It is a reinforcement of the security situation, and the expense of readability of information [9, 15, 21].

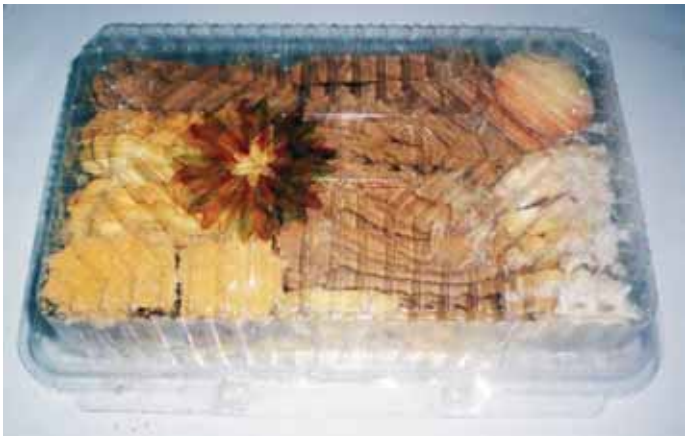


Fig. 8 – Flexographic translucent packaging with transparent IR Security graphics

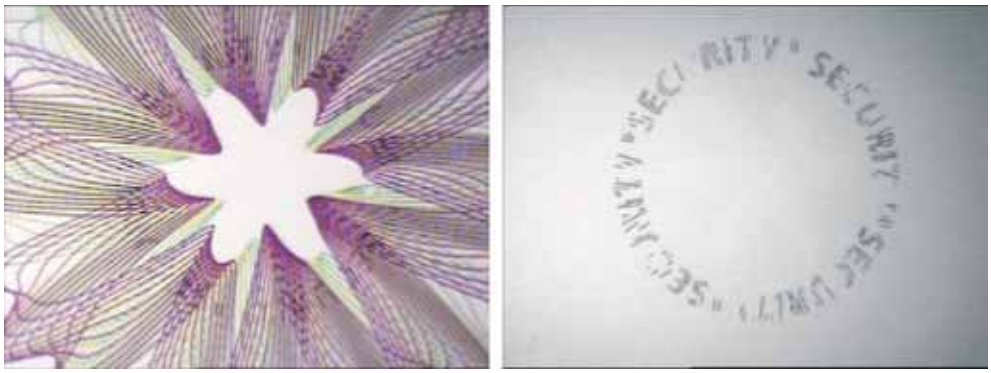


Fig. 9 a,b – Rosette with infrared information

Increasing requirement for flexible packaging is forcing the design of transparent graphics, so packaged product can be seen with bare eyes. Protected food products and belonging packaging materials are defined and regulated by strict international laws and regulations. IRD technology proves to be very successful in this area. The reasons are based on proven practices that do not need some new dyes, some "special colors" that are not approved for food packaging. Secondly, flexible packaging is done in millions of copies, so that each changeover print is unacceptable. IRD is a new practice in this area, which introduces security for publishers, printers and consumers. Dye manufacturers of are not offering IR dyes, neither colorants that have double status as twin pairs. Authors of IRD have set recipes for making colors for multiple states in the spectral range 400-1000 nm. The result is many spot colors, that have certain Z value, so independent designers can enter in the Infra protective area.

4.3. Postage stamps

IRD system is applied on a postage stamp "Croatian Accession to the European Union" by Jane Žiljak Vujic. Printing is carried out in offset with the process V, M, Y, K dyes, on the protected stamps paper [22].

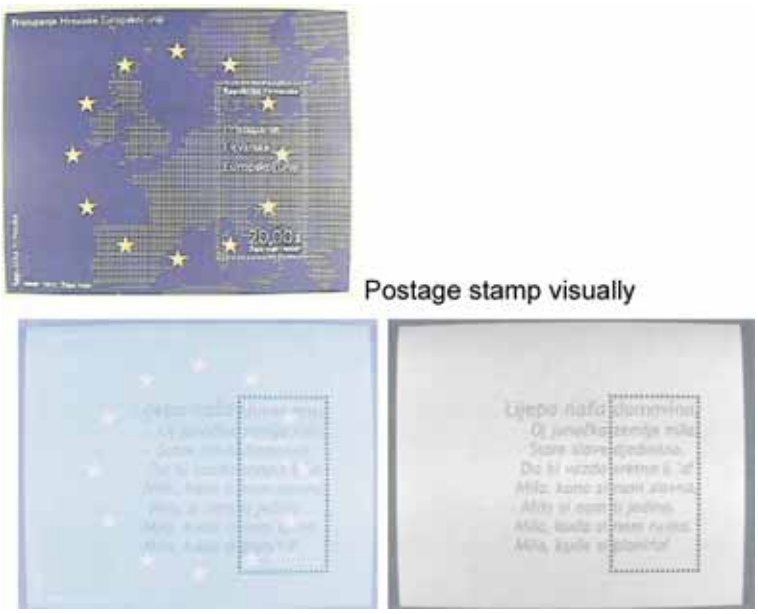


Fig. 10 a,b – Postage stamps: 715 nm, 1000 nm

The visual design is composed of Europe contour with yellow dots and yellow stars. Inserted perforated stamp carries information of purpose brands. Infrared design for 1000 nm consists the text of the national anthem, "Our Beautiful ..." Barrier scanning at 715 nm has a much weakened yellow and magenta, but enough to see 12 white stars in the rest of cyan and responds to 750 nm. The text of the national anthem is carbon black processing component. Stars are not recognized at 1000 nm, when cyan dye is no longer responding.

5. Z VALUE

Flora, fauna and minerals absorb in NIR in different ways. We use these properties to study the phenomenon of camouflage – Hiding in nature. We developed models staining of cotton, silk and leather, to hide them in a visual sight, and hide from

infrared lights equipped on night vision devices. ZRGB camera was developed that filtrates NIR in daylight, for example, the solar NIR component. Colored clothing must be successfully invisible at night and day in two spectra: V and Z. Paint subordinate to twins dyes. It is added the ability to recognize special occasions. Labelling for special purposes; hidden character to be recognized "on demand". The solution is in twins dyes that can handle multiple planned information. Painting dyes hold their own Z values. (Table 1). The values for tempera paintings with third each color has a value of grey tones. Z values and the K value for tempera painting dyes are weakly correlated. Otherwise, painters are never using "gray value" of their colors. K value is used in practice only in black / white photography. Measurements of Z values helps artists in orientation when creating double V / Z images. In nature, the more pronounced the agreement or disagreement, i.e. holds the correlation of gray tone and Z values. Especially is observed flora, especially fauna.

Tablica 1. Connect temtere (Slika 3.)

Names of dyes / color	RGB 0 d 255	Z (%)	K(%)
Grass Green	10, 148, 36	53	50
Cian blue	19, 84, 236	28	70
Carmine	252, 33, 63	13	56
Burnt sienna	213, 55, 43	48	59
Magenta	213, 53, 39	19	57
Yellow	253, 247, 0	18	3
Black	33, 25, 46	85	90
Ochre	233, 179, 9	24	26
Orange	253, 111, 0	20	42
Dark Green	15, 106, 153	60	64
Ultramarine	12, 12, 198	62	95

5.1. FLORA IN VZ SPECTRUM

We recognize and distinguish nature in RGB and Z state. Recording in range 400-1000 nm spectral lets us watch four subsectors: red, green, blue, infrared. ZRGB camera [5], was developed that has two parts. The first captures the V state and the other Z state. Call it the "dual recording" and "dual range" although there are all three RGB fields on their separate places. We strongly suggest not using term "four-channel spectrum."

Fig. 11 a,b – Flora in the visual and infrared spectrum

Figure 11 represents the flora image, flowers from the garden in bright sunny day. The resulting contrasts are the darker and lighter areas. Figure in the NIR allows measuring the Z value of each part of the flora and mineral coverage at the same pixel position. It's a start to create a new base of informations about the state of nature, in a more precise, extended description.

Fig. 12 a,b – Flora in V and Z spektrum on 1000 nm

Leaves refused the NIR light. Where the shadow of the leaves, there is less sunlight in the RGB (400-1000 nm) also in near-infrared part of sunlight. RGB camera and Z camera these places recorded as dark areas. Onions, carrots, parsley, cabbage, all are equal in the NIR spectrum.

Fruits show the same properties as leaves in the NIR spectrum. Orange, banana, apple, tangerine have properties refusal NIR wavelengths. Pip of kiwi and pomegranate has a certain Z value.

Fig. 13 a,b – Fruits in the visual and infrared spectrum

Conventional reproduction does not care about the Z state of nature. Whether it be someone? Well, nobody, if we make all with RGB camera. And if you just want such reproduction that same experience just as RGB. If we make a military infrared camera then we would have to play a different [11]. When, for such, Z camera flora is "white", then her clothing should also be white when viewed with an infrared camera. Certainly, if the camouflage carried out in the area of live plant. No matter if it's a sunny day or infrared light source for night monitoring of the army. Around us in the city, hundreds of ZRGB cameras are observing us in the VZ "light absorption system.

The absorption of infrared light for many plants is investigated. Black flowers and red wine are "white" in Z point. It is the claim of the flora, there is no correlation in the absorption of the visual and infrared spectrum.

5.2. FAUNA IN V / Z SPECTRUM

The absorption of light from the fauna has other properties. Figure 11 shows an extreme example of domestic cats. Its black hair shows up as black in the infrared spectrum. A high absorbance in NIR. Other shades of hair also absorb NIR. The brown tones are brighter (top of the ear and the hair on the leg), and these areas show the IR contrast. It is the same with the hair when mixed in a variety of colors.

With ZRGB camera domestic animals, animals in ZOO and animals in wood and nature generally were examined. Against flora, in animal world correlation between visual and NIR spectrum exists [30].

Fig. 14 a,b – Visual RGB image and Z image of domestic cats

The colors in the animal world have their own interpretation in the gray scale. The gray images of animals are very similar to the gray image which is obtained from Z camera. It's just an indicator that there is no general correlation of RGB and Z sizes in nature, but exists partially.

5.3. Military camouflage clothing

Infrared thermal recordings are passive observing properties of matter in the area of about 10,000 nm. If you would just keep on passive observation of flora and fauna and the NIR, we would ask why, why are we doing? Today, camouflage equipment, clothes and weapons are hidden only in the daylight. Since observed with the NIR camera, it is necessary to extend the technology to camouflage coloring with the aim that camouflage is considered in two spectral regions. Access to camouflage equipment in animal's environment is different than in the surrounding of plants. For each situation, action, for targeted activity, a specific solution is needed. Graduations of visual and hidden in the NIR stretches from zero absorption values to completely dark parts Z response [20].

Fig. 15 a,b – Camouflage uniform with identification in inner part

The exterior side of camouflage uniform upon our recommendations has invisible qualities for military IR cameras. It is achieving the same properties as flora. The inside coating of the jacket (Figure 15) is marked with state coat of arms, that is not seen in the visual spectrum. It can be identified with the IR camera, when there is intended [27, 17].

Fig. 16 a,b – Black boots with black dye that responds as white in the Z spectrum

Looking at the reproduction with two cameras, two images are obtained, the same as they were recorded. These two pictures representing: “the expanded reality”. Reproduction of nature photography and paintings develops new task. Graphic reproduction must have the same dual properties as it has the nature whose reproduction is presented. Let reproduction contains same properties in the visual and infrared spectrum. At the same place, two printed images whose source is the nature, are recorded in the two spectra: Nature shows two images (V and W) arising from the same recording of nature with two cameras, accepting two different states [28, 29,30, 31] .

6. New V / Z technology for graphic reproduction

Looking at the reproduction with two cameras, two images are obtained, the same as they were recorded. These two pictures representing: “the expanded reality”. Reproduction of nature photography and paintings develops new task. Graphic reproduction must have the same dual properties as it has the nature whose reproduction is presented. Let reproduction contains same properties in the visual and infrared spectrum. At the same place, two printed images whose source is the nature, are recorded in the two spectra: Nature shows two images (V and W) arising from the same recording of nature with two cameras, accepting two different states [28, 29,30, 31, 32] .

Z value [4] for leaves, animal hair, bark of trees, have been introduced in order to be able to establish an algorithm for the realization of printing forms, that adequately represent the expanded VZ spectrum, absorption and reflection of light in nature. We have introduced a numerical Z value as a measure of light absorption, for use throughout our environment: Proving originality in the process of creating a artwork. V / Z system is a new area of flora, fauna and minerals research. If there is no general correlation between RGB and Z values, this suggests that each image can be incorporated into RGBZ reproduction. IRD ® system is merging two independent graphics, and thus IRD ® becomes the new technology in security printing, with special emphasis for designing camouflage clothing. To graphic artists and physicists remains the task of finding a method of creating dyes and V/Z specification, which are simulating V and Z informations obtained from nature.

7. AWARDS

In this paper, it should be emphasized, that the IRD ® is Croatian brand. IRD ® is originated in Croatia, and presented worldwide. So far, the IRD ® achieved sixty gold, diamond, and special medals [10], in London, Pittsburgh, Moscow, Cardiff, Seoul, Taipei, Geneva, Budapest, Celje, and elsewhere. National Award for Science, awarded for the discovery in 2010th. The award from Pittsburgh, Award for Scientific merit was obtained in the year 2011. There are also prizes in Croatia: Golden kuna in 2011; Nikola Tesla Award in 2011; City of Zagreb Award 2010, from city mayor M. Bandic in 2011; Award Slavoljub Penkala in 2011. Invited lectures were held in several Universities in India, Greece, Austria, Bosnia and Herzegovina and Serbia.

IRD was presented at conferences and international meetings: IARIGAI Sweden, BIS Englska, INPEX – the U.S., inventions Geneva – Switzerland, CIGIF – Korea, ARHIMED – Russia, AGEP – Russia, IIFME – Kuwait, IENA Germany, IEIS – Slovenia, IWIS – Poland , Gwin – England, IASI / EUROINVENT – Romania, MARS,-Malaysia, AGEP, Moldova 2011, IFIA – Hungary, TIIS – Taiwan, INST – Taiwan, INOVA Croatia, NEAP – India, I-CAT India (www.infraredesign.net)

On the occasion of the discovery of new methods in the security printing, PIRA (London) organized its annual congress in Zagreb, 2011, dated on actual birthday of Ivana Žiljak, and what was the first announced of this discovery in the year 2007.

Comprehensive view of creation, development, awards in the past six years on INFRAREDESIGN-in is given in the last article on the 15th invitee International Conference on printing, design and graphic communications Blaz Baromić [16]. At the same conference there are presented papers on the IRD ® approach of inking leather and footwear [17]. Multi-year experiments with stochastic solutions for the packaging of hiding images are shown in the works of Jana Žiljak and Dora Perčić [18].

8. CONCLUSION

It's been six years since INFRAREDESIGN ® was discovered, integrating observing and study of wavelength area from 400 to 1000 nanometres, in a single graphic, printing and information system. We measure the absorption and reflection of sunlight from the flora, fauna and minerals, including ultraviolet, visual and near infrared spectrum. This knowledge is needed to understand the nature beyond visual range. Furthermore, we intended for active painting with purpose. Making camouflage equipment with hiding in visual and infrared spectrum. We defined new types of protection in many areas that are under attack from counterfeiters. It is true that

around us are many IR cameras. The intention of the IRD is to be fully managed by identifying the environment in the expanded spectrum. That covers the domain of surveillance cameras, and that means in visual and infrared spectrum at the same time, both by day and night.

Dyes are studied for application for textiles, leather industry and printing graphic technology; In the visual arts, particularly in fashion and painting; Expanded reproduction techniques with the aim of reproduction paintings that have the same characteristics as the originals, especially if it is an art work made as a double V / Z image. The visual spectrum is for the eyes V; from 400 to 700 nm. An additional part of 300 nm is following the new Infra – graphic Z system.

The unit of measurement for absorption of light at the end of that area: named Z parameter was introduced. Graphic artists and physicists must overcome in reproducing camouflage equipment with CMYKF dyes, so that reproduction shows the same condition as recorded RGBZ environment. Discovery of color management for the extended spectrum has wide variety of applications in the future: security graphics, quality control prints, camouflage clothing, textile design with hidden marking, artistic painting.

References

1. I. Žiljak,K. Pap, J. Žiljak-Vujić, Infraredesign Zagreb : Fotosoft (2008).
2. I. Žiljak, K. Pap, J. Žiljak-Vujić, Infrared security graphics .Zagreb : FotoSoft (2009).
3. K. Pap, I. Žiljak, I., J. Ž. Vujić, Proces Color Management for Producting Double Images, (2008), Annual of the Croatian Academy of Engineering, pp. 395-410.
4. V. Žiljak K. Pap, I. Žiljak-Stanimirović, J. Žiljak-Vujić, (2012.). Managing dual color properties with the Z-parameter in the visual and NIR spectrum, Infrared physics & technology, 55 (2012) 326-336.
5. V. Žiljak, K. Pap, I. Žiljak Stanimirović, Development of a Prototype for ZRGB Infraredesign Device, Technical Gazette, 18 (2011) 153-159.
6. [www.projectina.ch]
7. K. Pap, I. Žiljak, J. Žiljak-Vujic, Image Reproduction for Near Infrared Spectrum and the Infraredesign Theory, Journal of Imaging Science and Technology, 54 (2010) 10502-1-10502-9.
8. V.Žiljak, K. Pap, I. Žiljak, CMYKIR SECURITY GRAPHICS SEPARATION IN THE INFRARED AREA, Infrared Physics and Technology, 52. (2009) 62-69.
9. M. Friščić, J. Žiljak Vujić, V. Žiljak, K. Pap, "Invisible prints on transparent materials with flexo printing, PRINTING & DESIGN 2013, Department of Graphical Engineering of the Croatian Academy of Engineering (2013) pp. 151 – 157.
10. http://infraredesign.fotosoft.hr/
11. I. Žiljak, K. Pap,J. Žiljak Vujic, INFRARED DESIGN ON TEXTILES AS PRODUCT PROTECTION, // TEKSTIL, 58 (2009) 239-253.
12. A.M. Politis, M. Rudolf, J. Žiljak Vujić, CMYKIR SEPARATION OF TWO PORTRAITS FOR POSTAGE STAMP DESIGN", PRINTING & DESIGN 2013, Department of Graphical Engineering of the Croatian Academy of Engineering (2013) pp. 7-10.

13. M. Matas, A. Rajendrakumar, J. Žiljak Vujić, A. Hoić, The role and significance of a designer in postage stamp design with Infrared Graphics, PRINTING & DESIGN 2013, Department of Graphical Engineering of the Croatian Academy of Engineering (2013) pp. 11 -13.
14. I. Čaljkušić, A. Hoić, J. Žiljak Vujić, The reproduction of a visual work of art with its visual and infrared state, PRINT (2013).
15. M. Friščić, I. Žiljak Stanimirović, J. Žiljak Vujić; INFRARED TEHNOLOGIJA U FLEKSOTISKU SA SPOT BOJAMA, 16. međunarodna konferencija tiskarstva, dizajna i grafičkih komunikacija Blaž Baromić, (2012) pp. 503 – 512.
16. I. Žiljak Stanimirović, INFRAREDESIGN® TISKARSKA TEHNOLOGIJA KOJA OBJEDINJUJE VIZUALNI I INFRACRVENI SPEKTAR, 16. međunarodna konferencija tiskarstva, dizajna i grafičkih komunikacija Blaž Baromić, (2012) pp. 14-37.
17. I. Žiljak Stanimirović, J. Akalović, J. Žiljak Vujić , DESIGN AND PRINT OF HIDDEN INFORMATION ON LEATHER WITH SPOT INKS THAT HAVE KNOWN Z FACTOR" 16. međunarodna konferencija tiskarstva, dizajna i grafičkih komunikacija Blaž Baromić, (2012) pp. 479 – 490.
18. J. Žiljak Vujić, D. Perčić, INFRAREDESIGN® NA AMBALAŽI U FARMACEUTSKOJ INDUSTRIJI, 16. međunarodna konferencija tiskarstva, dizajna i grafičkih komunikacija Blaž Baromić,(2012) pp 38 – 46.
19. B. Hlevnjak, D. Kinert, V. Rismondo ml.; Nada Žiljak, INFRAREDART, Fotosoft, Zagreb (2012).
20. I. Ž. Stanimirović, J. Ž. Vujić, N. L. Stanić, Marking of the camouflage uniform for visual and near infrared spectrum", TTEM 8 (2013) 920-926.
21. I. Žiljak Stanimorović, J. Žiljak Vujić, B. Morić, M. Rudolf, Security printing with colorant control in the UV, visual and INFRARED spectrum; TTEM 8 (2013) 480-485.
22. J. Žiljak Vujić, B. Morić , M. Rudolf, M. Friščić, Postage Stamps with hidden information in security Z values, TTEM 8 (2013) 1466-1473.
23. V. Žiljak, K. Pap, I. Žiljak, INFRARED HIDDEN CMYK GRAPHIC", The Imaging Science Journal 58. (2009) 20-27.
24. M. Barisic, N. Mrvac, M. Milkovic, New rationalization methods of rotary printing in the field of planographic printing", TTEM 8 (2013) 513 – 523.
25. A. Rajendradrakumar, V. Žiljak, Invisible pics hit newspaper", RIND Survey, Rs.40.00, 32 (2011) 4-6.
26. J. Žiljak Vujić, I. Žiljak Stanimirović, O. Međugorac, HIDDEN INFORMATION IN VISUAL AND INFRARED SPECTRUM, Informatologija 45 (2012) 96 – 102.
27. V. Žiljak, J. Akalović, J. Žiljak-Vujić, UPRAVLJANJE BOJILIMA NA KOŽI U VIZUALNOM I INFRACRVENOM SPEKTRU. // Tekstil : časopis za tekstilnu tehnologiju i konfekciju. 60 (2011) 355-363.
28. J. Ž. Vujić, A. Agić, N. L. Stanić, Picture information in expanded color management from visual to near infrared spectral domain", TTEM 8 (2013) 942-950.
29. D. Agić, I. Stanimirović Žiljak, A. Agić, N. Stanić Loknar, Degradation of Dual Image for Visual and near Infrared Spectrum at repeated CMYK/RGB Rendering; Journal of Graphic Engineering and Design 4 (2013) 13-16.
30. V.Žiljak,Grafika u spektru od 400 do 1000 nanometra; Blaž Baromić 2013, Vilko Ž, Ivana ?
31. I. Žiljak Stanimirović, J. Žiljak Vujić, M. Matas, INFRARED COLORANTS AS TWINS FOR SECURITY PRINTING OF DOCUMENTS AND SECURITIES, 45th Conference of the International Circle of Educational Institutes for Graphic Arts Technology and Management (IC), Toronto, Canada (2013).
32. M. Friščić, O. Međugorac, L. Tepeš, D. Jurečić, Invisible information on the transparent polymer food packaging with Infra V/Z technology, TTEM 8 (2013) 1512 -1519.

List of Reviewers for papers presented at the 2nd International Symposium "Vera Johanides"

1. Prof. Sunčica Beluhan, Ph.D., Faculty of Food Technology and Biotehnology, University of Zagreb
2. Prof. Marijan Bošnjak, Ph.D., member emeritus of HATZ
3. Prof. Mladen Brnčić, Ph.D., Faculty of Food Technology and Biotehnology, University of Zagreb
4. Prof. Frane Delaš, Ph.D., Faculty of Food Technology and Biotehnology, University of Zagreb
5. Prof. Zoran Herceg, Ph.D., Faculty of Food Technology and Biotehnology, University of Zagreb; collab. member of HATZ
6. Prof. Daslav Hranueli, Ph.D., Faculty of Food Technology and Biotehnology, University of Zagreb; member of HATZ
7. Prof. Damir Ježek, Ph.D., Faculty of Food Technology and Biotehnology, University of Zagreb; collab. member of HATZ
8. Prof. Jasna Kniewald, Ph.D., member emeritus of HATZ
9. Prof. emer. Zlatko Kniewald, Ph.D., member emeritus of HATZ
10. Prof. Želimir Kurtanjek, Ph.D., Faculty of Food Technology and Biotehnology, University of Zagreb; member of HATZ
11. Prof. Vesna Lelas, Ph.D., Faculty of Food Technology and Biotehnology, University of Zagreb; member of HATZ
12. Prof. Biserka Nagy, Ph.D., collab. member of the Croatian Academy of Science and Arts
13. Prof. Vlasta Piližota, Ph.D., Faculty of Food Technology, University of Osijek; member of the Croatian Academy of Science and Arts
14. Prof. Božidar Šantek, Ph.D., Faculty of Food Technology and Biotehnology, University of Zagreb; collab. member of HATZ
15. Prof. Branimir Šimić, Ph.D., Faculty of Food Technology and Biotehnology, University of Zagreb
16. Prof. Jagoda Šušković, Ph.D., Faculty of Food Technology and Biotehnology, University of Zagreb; member of HATZ
17. Prof. Renata Teparić, Ph.D., Faculty of Food Technology and Biotehnology, University of Zagreb
18. Prof. Srećko Tomas, Ph.D., Faculty of Food Technology, University of Osijek; member of HATZ
19. Prof. Branko Tripalo, Ph.D., Faculty of Food Technology and Biotehnology, University of Zagreb; member of HATZ
20. Prof. Žaneta Ugarčić, Ph.D., Faculty of Food Technology, University of Osijek; member of HATZ
21. Prof. Vesna Zechner Krpan, Ph.D., Faculty of Food Technology and Biotehnology, University of Zagreb

List of HATZ Members participating in the realisation of Annual 2013.

1. Prof. Marijan Bošnjak, Ph.D., member emeritus of HATZ
2. Prof. Jasna Franekić, Ph.D., member of HATZ
3. Prof. Zoran Herceg, Ph.D., Faculty of Food Technology and Biotehnology, University of Zagreb; collab. member of HATZ
4. Prof. Predrag Horvat, Ph.D., Faculty of Food Technology and Biotehnology, University of Zagreb; collab. member of HATZ
5. Prof. Daslav Hranueli, Ph.D., Faculty of Food Technology and Biotehnology, University of Zagreb; member of HATZ
6. Prof. Damir Ježek, Ph.D., Faculty of Food Technology and Biotehnology, University of Zagreb; collab. member of HATZ
7. Prof. Jasna Kniewald, Ph.D., member emeritus of HATZ
8. Prof. emer. Zlatko Kniewald, Ph.D., member emeritus of HATZ
9. Prof. Želimir Kurtanjek, Ph.D., Faculty of Food Technology and Biotehnology, University of Zagreb; member of HATZ
10. Prof. Vesna Lelas, Ph.D., Faculty of Food Technology and Biotehnology, University of Zagreb; member of HATZ
11. Prof. Milena Mandić, Ph.D., Faculty of Food Technology, University of Osijek; member of HATZ
12. Prof. Vladimir Mrša, Ph.D., Faculty of Food Technology and Biotehnology, University of Zagreb; collab. member of HATZ
13. Prof. Srđan Novak, Ph.D., Faculty of Food Technology and Biotehnology, University of Zagreb; member of HATZ
14. Prof. Dragan Primorac, Ph.D., School of Medicine, University of Split; University of New Haven, USA; honorary member of HATZ
15. Prof. Božidar Šantek, Ph.D., Faculty of Food Technology and Biotehnology, University of Zagreb; collab. member of HATZ
16. Prof. Marin Soljačić, Ph.D., Physics Department, MIT, USA; corresponding member of HATZ
17. Prof. Drago Šubarić, Ph.D., Faculty of Food Technology, University of Osijek; member of HATZ
18. Prof. Jagoda Šušković, Ph.D., Faculty of Food Technology and Biotehnology, University of Zagreb; member of HATZ
19. Prof. Srećko Tomas, Ph.D., Faculty of Food Technology, University of Osijek; member of HATZ
20. Prof. Branko Tripalo, Ph.D., Faculty of Food Technology and Biotehnology, University of Zagreb; member of HATZ
21. Prof. Žaneta Ugarčić, Ph.D., Faculty of Food Technology, University of Osijek; member of HATZ
22. Prof. Vilko Žiljak, Ph.D., Polytechnic of Zagreb, member of HATZ

List of Supporting Institutions and Donators

Zaklada je osnovana s ciljem promicanja biotehnologije, prehrambene tehnologije, nutricionizma i zaštite okoliša, a svake godine dodjeljuje stipendije, potpore i nagrade redovitim i najboljim studentima koji svojim rezultatima tijekom studija na preddiplomskim/diplomskim i doktorskim studijima PBF-a mogu pridonijeti razvoju gospodarstva Republike Hrvatske.

http://www.biotehnicka-zaklada.hr

HRVATSKO DRUŠTVO ZA BIOTEHNOLOGIJU
CROATIAN SOCIETY OF BIOTECHNOLOGY
KROATISCHE GESELLSCHAFT FÜR BIOTECHNOLOGIE
SOCIÉTÉ CROATE DU GENIE BIOLOGIQUE

We acknowledge the assistence
of Ministry of Science, Education and Sports
of the Republic of Croatia
in publishing this book.